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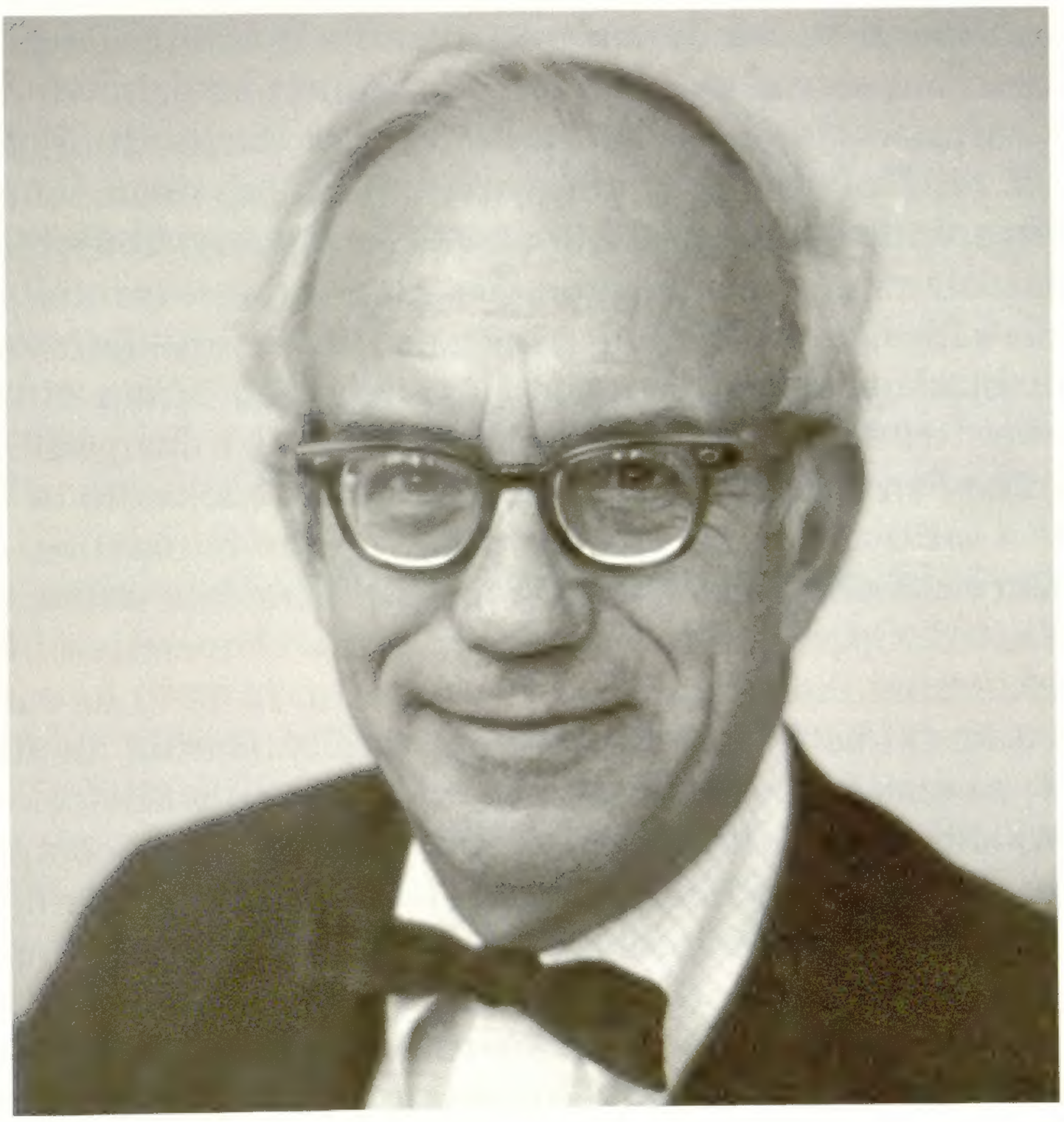
Obituary: Rolla Milton Tryon, Jr. (1916–2001)



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Rolla Tryon, a member of the American Fern Society since 1932 and one of the twentieth century's most eminent students of pteridophytes, was born on August 26, 1916 in Chicago, Illinois. His father, a professor of American history and education at the University of Chicago, maintained a summer cottage in Chesterton, Indiana in addition to his home in Chicago. Rolla's fascination with ferns and fern allies developed during boyhood forays from that Ches-

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The photograph was taken by Dr. Walter H. Hodge in Mexico City in December, 1972 and was made available by the Hunt Institute for Botanical Documentation.

terton cottage into sand dune habitats along Lake Michigan in the northwest of Indiana. At the age of 18, he published his first paper, relating his observations on *Osmunda* plants in the Indiana Dunes (see complete bibliography below). As a boy, Rolla was greatly influenced by, and in turn influenced, Charles Deam (author of the 1940 *Flora of Indiana*), advising Deam about fern species he had found in the dunes area. When a doubting Deam appeared at the cottage door one day asking to meet Rolla and to be shown these ferns *in situ,* he was surprised to learn that Rolla was not the adult of the family but a mere boy of 14. Thus began a productive friendship documented in Rolla's correspondence with Deam from May, 1935 to January, 1953. All the penny postcards and letters he received from Deam have been carefully maintained in one of Rolla's files, now archived at Indiana University—fascinating reading. Rolla's insatiable boyhood appetite for ferns got him into a bit of trouble at home, however, when his father learned that he had charged Bower's three volumes on *The Ferns* to his account at Brentano's bookstore in Chicago.

Rolla built a solid academic superstructure on the foundation of these boyhood experiences. Among his academic accomplishments were an A.A. degree in 1935 and a B.S. degree in 1937, both from the University of Chicago, and a Ph.M. in 1938 from the University of Wisconsin. In 1940 he earned an M.S. and in 1941 a Ph.D., both from Harvard University. During his days as a Harvard student, he contracted malaria in South Carolina while collecting plants for M. L. Fernald, and during the war-torn year following completion of his Ph.D. he served as a lab technician in the U. S. Chemical Warfare Service at Massachusetts Institute of Technology. His father thought he should follow his Ph.D. in botany with another, this time in chemistry, so that he could earn a living, but instead Rolla became an Instructor in Botany first at Dartmouth College, then at the University of Wisconsin before becoming an Assistant Professor in Botany at the University of Minnesota in 1945. While an Assistant Professor at the University of Wisconsin, Rolla met Alice Faber. Their marriage in 1945 initiated not only a happy and enduring domestic partnership but also a research synergism whose productivity has nourished pteridologists throughout the world.

In 1947 Rolla became Associate Professor in Botany at Washington University, St. Louis and Assistant Curator of the herbarium of the Missouri Botanical Garden, positions he held to 1957. During this appointment, he and Alice were the original organizers of the Missouri Botanical Garden's annual Systematics Symposium, whose 48th meeting was held 12–13 October, 2001. This highly successful annual meeting has received continuous support from the National Science Foundation from its second year (1954) to the present (with the lapse of a single year). From 1946 to 1957 Rolla served as curator and librarian of the American Fern Society's library and herbarium, responding to members' requests for loans of materials. That herbarium and library was subsequently entrusted to Warren H. Wagner at the University of Michigan. Following a year as Research Associate at the University of California, Berkeley, Rolla went to the Gray Herbarium of Harvard University as Associate Curator and Curator of Ferns in 1958 and became Curator of the Gray Herbarium in 1967. Rolla

and Alice traveled the world extensively, attending international meetings, conducting field work, studying specimens at major herbaria in the Americas, Europe, and Africa, and conducting field courses on the ferns. In addition to other services to professional societies, Rolla served for many years as Associate Editor of *Rhodora* and the *American Fern Journal,* as Associate Editor of *Brittonia* (1961–1964), as Editor-in-Chief of *Rhodora* (1977–1982), and as President of the New England Botanical Club and the American Fern Society.

A framed photograph of his revered mentor, Charles A. Weatherby (see *American Fern Journal* 40[1] for a remarkable series of papers honoring this unusually respected and beloved botanist), was always prominently displayed on Rolla's desk at Harvard, undoubtedly inspiring his own welcoming, patient, and supportive response to all who entered his office seeking counsel. In 1970 Rolla initiated an annual New England Fern Conference at Harvard Forest. For 20 years this provided a stimulating intellectual setting in which students of fern biology discussed and developed their ideas. In 1972 he became Professor of Biology at Harvard University, holding both the Curatorship and Professorship until his retirement in 1987. He remained at Harvard as Professor Emeritus from 1987 to 1989 when he moved to the University of South Florida in Tampa as Adjunct Professor, bringing with him his extensive library of fern and biogeographic literature. To mark the retirements of Alice and Rolla Tryon from Harvard, a festschrift of 13 papers plus introduction was published in their honor in the *Annals of the Missouri Botanical Garden* (vol. 77: 225–339. 1990).

At the University of South Florida, Rolla and Alice helped found the Institute for Systematic Botany and endowed the Tryon Lecture Series that brings several internationally known botanists to the university each year. In their research-active office on the Tampa campus, he and Alice continued their pteridological work, as his following bibliography indicates.

Rolla Tryon's publication list exceeds 100 titles and includes a great breadth of topics. Papers ranged from articles on pteridophytes for the 1943 *Encyclopedia Britannica* to a glossary of terms relating to the fern leaf, discussions of the history of pteridology and fern classification, a remembrance of his graduate mentor and counselor Charles A. Weatherby, discussions of the formalities of fern nomenclature, and many book reviews. His monographs and revisions focused mostly on ferns but also included angiosperms *Convolvulus* and *Elymus.* Signal among these were his revisions of *Pteridium, Doryopteris,* the *Selaginella rupestris* group, American *Notholaena,* and the Cyatheaceae. His papers on fern biogeography began with *Doryopteris* in 1944, matured in his exposition of geographic speciation in *Selaginella* in 1971, and continued to his and Alice's 1999 discussion of the phytogeography of eastern North American ferns (honoring Ching Ren-Chang). Floristic and taxonomic notes on ferns ranged from simple observations of growth forms and hybrids to elucidations of complex taxonomic and nomenclatural issues. For his 1955 publication on the taxonomy of cycads (coauthored with students in his Washington University class) he was awarded the 1956 Robert Montgomery award of the Fairchild Tropical Garden for distinguished achievement in the world of palms

and cycads. At the time of his death, he had a book review in press in *Rhodora* and a paper in press in *Bradea* (coauthored with his former student Paulo Windisch).

In addition to his numerous papers on ferns and other topics, Rolla is notable for his books. Among these are two editions of his *Ferns and Fern Allies of Wisconsin* (1940, 1953) and *Ferns and Fern Allies of Minnesota* (1954, 1980). He is renowned for his knowledge of the ferns of Peru, first expressed in his 1964 *Ferns of Peru* (250 pages in the *Contributions from the Gray Herbarium of Harvard University*). This treatment was updated and completed in six subsequent parts entitled *Pteridophyta of Peru* between 1989 and 1994, mostly coauthored with Robert Stolze but with several portions contributed by other pteridological specialists. His monumental 1982 book *Ferns and Allied Plants with Special Reference to Tropical America,* coauthored with Alice Tryon, is an encyclopedic treatment of this subject that continues to stimulate new research, as does his treatment of Pteridaceae, with Alice Tryon and Karl Kramer, in volume 1 of *Families and Genera of Vascular Plants* edited by K. Kramer and P. S. Green.

Rolla's kindly and perceptive mentoring and his outstanding contributions to our knowledge of ferns is signaled by having the following four fern taxa named in his honor.

1) *Asplenium tryonii* Correll. In describing this species, Donovan Correll (1961) said "It is a pleasure to name this species for Dr. Tryon, who has always been most gracious in helping his fellow-workers with their never-ending problems in the study of ferns." Known only from Chihuahua, Mexico, this species was further discussed and illustrated in *Ferns and Fern Allies of Chihuahua* by Knobloch and Correll (1962).
2) *Alsophila tryonorum* Riba. The eminent Mexican pteridologist Ramón Riba (see American Fern Journal 90:112–118, 2000) stated that "this species is named after Dr. Rolla M. Tryon and Dr. Alice F. Tryon for their contributions to the taxonomy of the ferns" (Riba, 1967). The plural specific epithet recognizes the close professional relationship between this highly productive research team. This tree fern species is now known as *Trichipteris tryonorum* (Riba) R. Tryon following its transfer by Rolla in his 1970 paper on the classification of the Cyatheaceae.
3) *Nephelea tryoniana* Gastony. "I am pleased to name this species for my mentor, Dr. Rolla M. Tryon, in recognition of his outstanding contribution to the understanding of the systematics and evolution of the family Cyatheaceae" (Gastony, 1973). Subsequent research by Conant (Conant and Cooper-Driver, 1980; Conant, 1983) revealed that this tree fern species is a reproductively stabilized diploid hybrid species that is now regarded as *Alsophila tryoniana* (Gastony) Conant.
4) *Tryonella* Pichi Sermolli. This new generic name was established by Pichi Sermolli (1974) "in honour of the eminent pteridologist R. M. Tryon, Jr., author of many important papers on ferns, who, *inter alia,* supported the distinction of the present genus from *Doryopteris,* though without giving it

a new name." This name is currently regarded as a synonym of *Doryopteris* by Tryon and Tryon (1982) and Tryon, Tryon, and Kramer (1990).

Among the doctoral graduate students he trained, Rolla counted the following (those with asterisks received their degrees from other institutions): Alice F. Tryon, *Karl Kramer, *Ramon Riba, Gerald Gastony, Lawrence Palkovic, David Barrington, David Conant, Paulo Windisch, *R. James Hickey, *Robbin Moran, Sonia Sultan, and Calvin Sperling. He also mentored Robert Stolze in his taxonomic revision of *Cnemidaria* at the Field Museum. Always available to his students, he modeled his supportive and insightful mentoring on his experiences with his own graduate mentor, Charles Weatherby. For this he has earned our love as well as our respect. His impact on his students, and their students, and their students is incalculable.

In 1978 Rolla M. Tryon, Jr. was elected to honorary membership in the American Fern Society, a special category of membership for persons who have made outstanding contributions to the study of ferns. In 1984 he received a Merit Award from the Botanical Society of America "In recognition of distinguished achievement in and contributions to the advancement of botanical science. Pre-eminently knowledgeable in matters of taxonomy and nomenclature, this foremost pteridologist is a perceptive student of phytogeography and of the evolutionary impact of the selective process during plant migration."

In addition to Rolla's botanical activities he was also highly skilled in running a family farm in Knox County, Indiana for many years. He visited the farm, overseeing its management, a few times each year. The extensive records he kept in managing crops and livestock illustrate his practical ability in managing business as well as scientific data.

On August 20, 2001, six days before his eighty-fifth birthday, Rolla Milton Tryon, Jr., left us to continue our work with the pteridophytes of the world, and to delight in them, without him. We do this fortified by his writings, the echoes of his encouraging words, and his everlasting example. He was the beloved husband of Alice Faber Tryon, the benefactor of countless students of pteridophytes, including many who never knew him personally, an inspiration and counselor to many collaborators and coauthors, the advisor of doctoral students, the teacher of innumerable undergraduates, and our dear friend and mentor. He will be deeply missed. He already is.

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Crystals Associated with the Intertracheid Pit Membrane of the Woody Fern *Botrychium multifidum*

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ABSTRACT.—CALCIUM-containing crystals have been found in the lumens of secondary tracheids in the rhizome of the woody fern *Botrychium multifidum*. These crystals are styloids with rough, pyramid- shaped ends. The crystals are usually single; however, conjoined or grouped crystals were also found. Crystal formation apparently has no constant relation to the pit membrane, but crystals of mature tracheids are often associated with the pit membrane or are located in the pit areas. Crystals were also located between the helical thickenings of the lumen walls. No crystal chamber or crystal sheath was found in association with the crystal body.

Crystals are a common feature in many plant tissues (Scurfield and Mitchell, 1973), and more than 1000 crystal producing woody plants, spanning 160 families, were described at the light microscopic level by Chattaway (1955, 1956). Scanning electron microscopy has allowed for more rapid identification of crystals in plant tissues and a clearer picture of their morphology (Scurfield and Mitchell, 1973). Although crystals in xylem tissue have been reported in the vessels of *Intsia* Thouars (Fabaceae; Hillis, 1996), *Torreya yunnanesis* C.Y. Cheng & L.K. Fu (Taxaceae; Kondo *et al.*, 1996), and *Polyalthia* Blume (Annonaceae; Scurfield and Mitchell, 1973), they are most commonly found in the xylem parenchyma, septate fibers, or vessel tyloses (Scurfield and Mitchell, 1973).

The formation of crystals by *Botrychium*, the only extant fern that produces wood (Gifford and Foster, 1989), has not been previously reported. During our studies of the torus-bearing pit membrane in the tracheid of *Botrychium multifidum* (S.G. Gmelin) Rupr., we discovered occasional instances of crystals associated with the pit membrane. This paper describes the morphology of these crystals as observed with SEM. The discoverey of these very small crystals was unexpected, and our exploration of them to this point has been strictly descriptive. However, in our discussion we explore several possible reasons for crystal formation in this wood.

MATERIALS AND METHODS

Rhizome samples of upright or orthotropous rhizomes of *Botrychium multifidum*. were collected by Dr. D. W. Stevenson (New York Botanical Garden, New York City, U.S.A.) from Plumas County, California and fixed in FPA. The collection site (elevation 2000 m) is rocky mountain soil at the edge of a meadow and the ground is frozen for much of the year. The samples were typical rhizomes selected as random samples and representative of the population. In

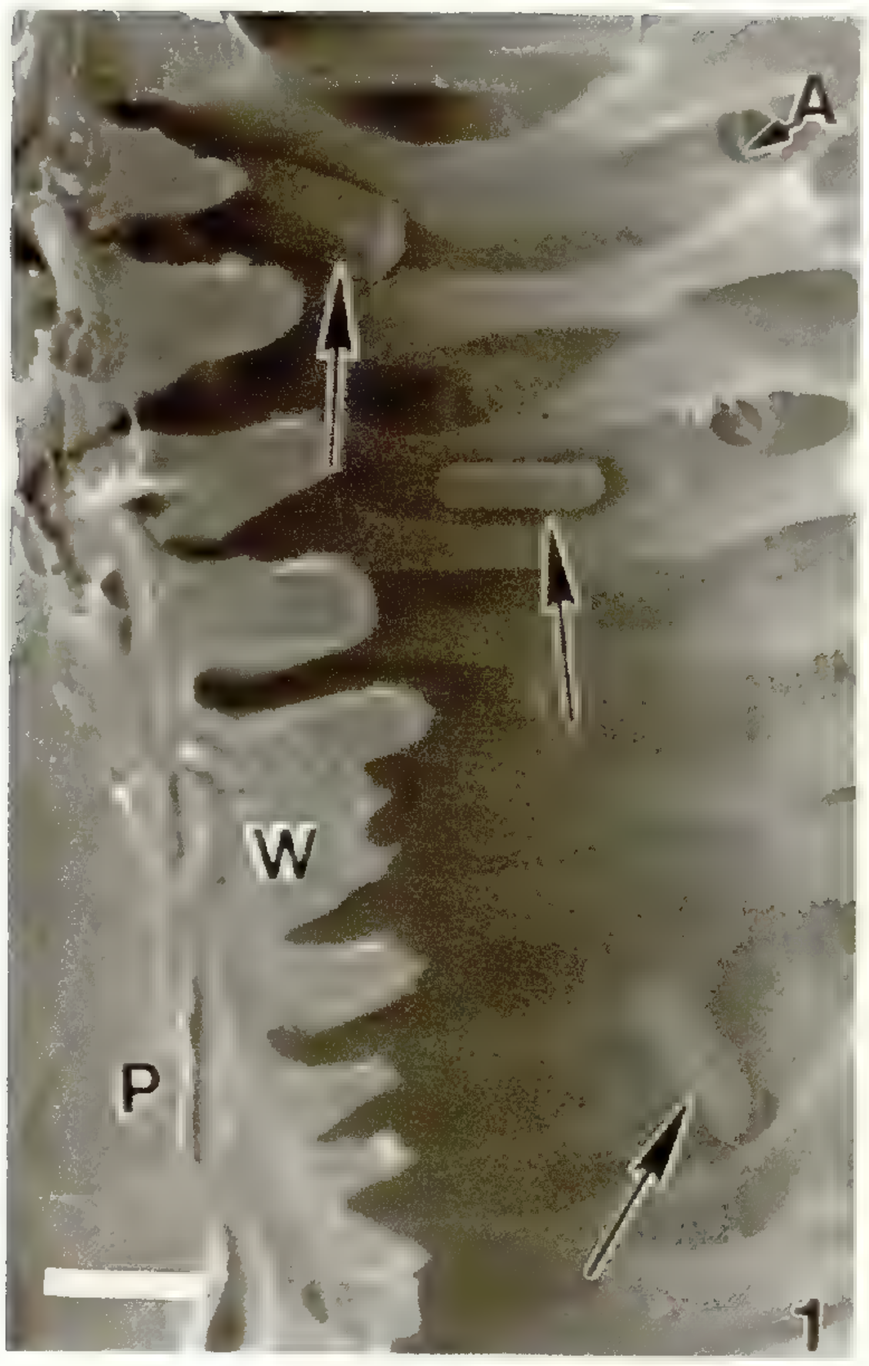
our lab, samples were cut transversely into 1–2 mm pieces that were placed into 50% ethanol and then dehydrated through a graded alcohol series. Samples then were cut into small wedges, placed into hexamethyldisilazane (HMDS) for 2 hours (Nation, 1983), and subsequently placed under a chemical hood overnight to dry. Dry samples were attached to aluminum stubs with double-sided sticky tape and coated with gold-palladium. For comparison purposes, samples of *Botrychium dissectum* Sprengel and *B. virginianum* (L.) Swartz from Lee County, Alabama were prepared in the same manner as *B.multifidum*. Specimens were viewed with a Zeiss DSM 940 at 5,10, or 15 kV. Qualitative element identification was performed using energy dispersive spectroscopy (Tracor Northern Micro Z II) coupled to the SEM.

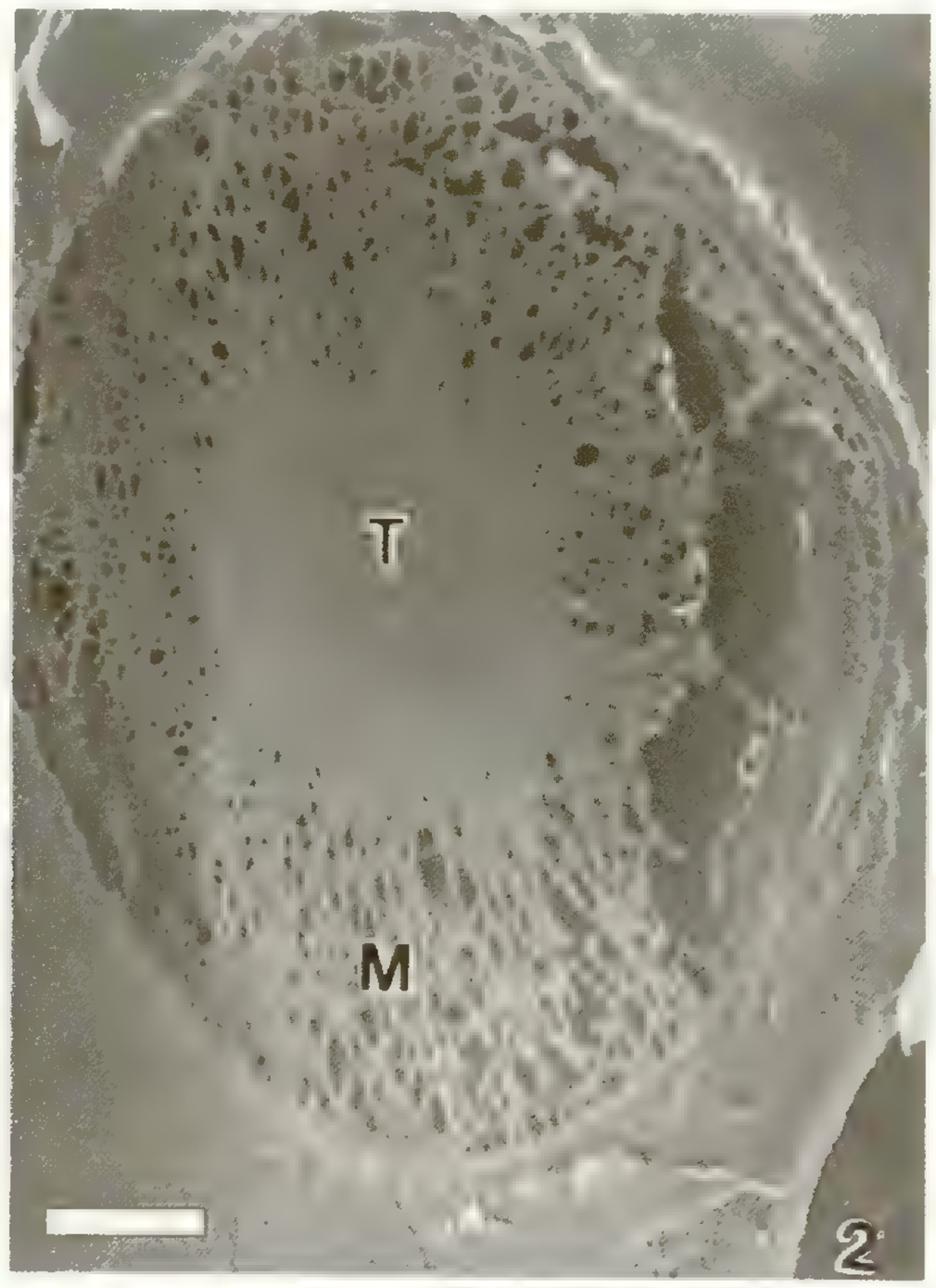
Results

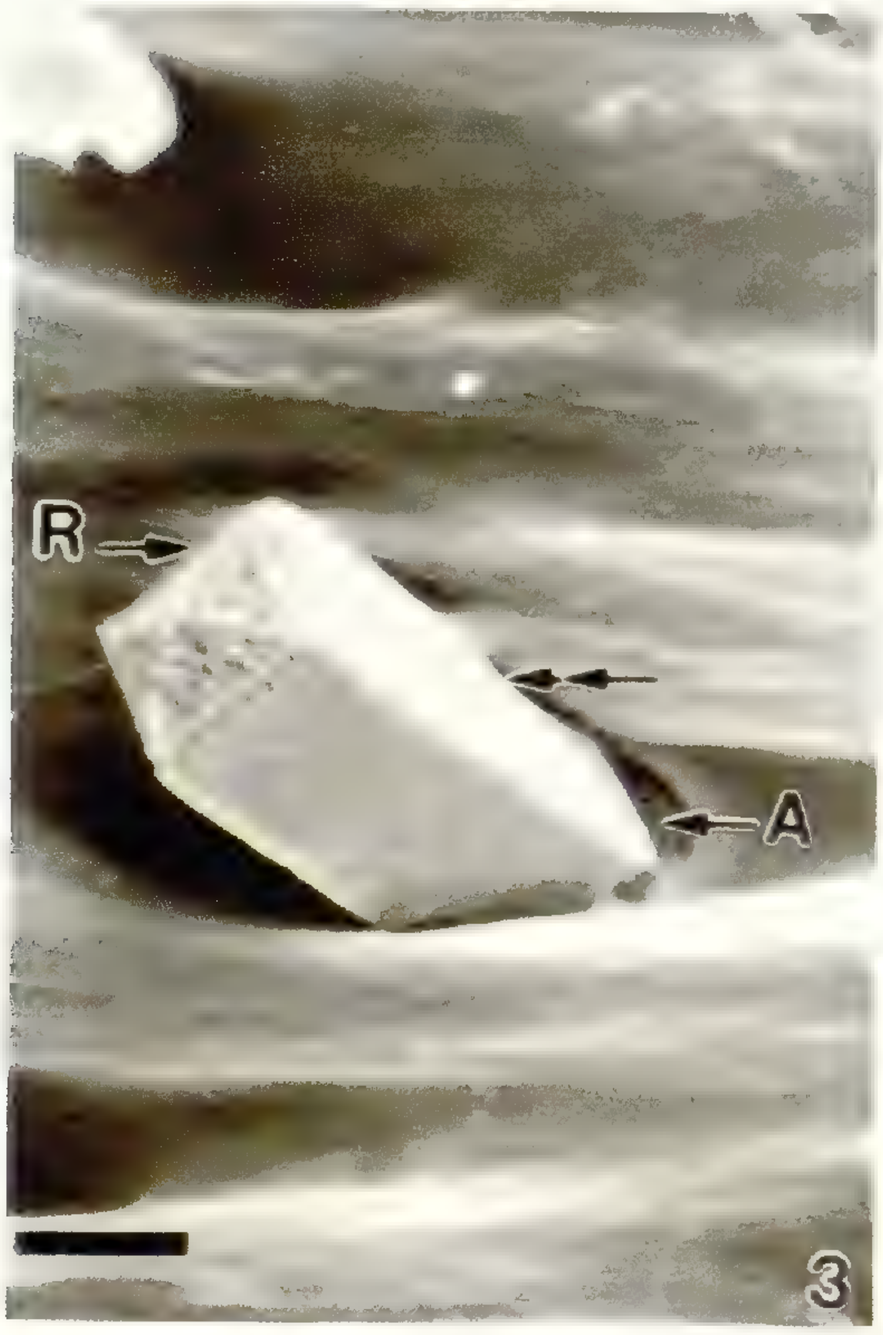
Secondary xylem tracheids of *Botrychium multifidum* contain helical wall thickenings and intertracheid circular bordered pits (Fig. 1). Thickenings, as seen in longitudinal section, are uniform neither in height nor in distance between gyres, and thickenings are sometimes branched (Fig. 1). The pit membrane is almost always differentiated into a torus and margo (Fig. 2). Microfibrils of the pit membrane are loosely woven in the margo region, but tightly woven in the torus. Tearing of the pit membrane was sometimes evident in the margo (Fig. 2). Crystals were found in association with torus-bearing pit membranes of tracheids (Fig. 11), as well as in tracheid lumen (Figs. 1, 3). These crystals were not apparent at the light level. Crystals associated with these tracheids are styloids (Frey-Wyssling, 1981; Carlquist, 1988); they are rectangular columnar with pyramidal ends. Intact crystals have columns that are four-sided and are smooth-surfaced. The pyramidal crystal ends consist of four equilateral triangles, although wedge-shaped ends also were observed (Fig. 4). Crystal ends, when visible, typically appeared to be rough (Fig. 3), although some crystals with smooth ends were observed (Fig. 4). Crystals ranged in size from 4.3 to 12 μm in length, and 1.14 to 2.4 μm in width (N = 12). The mean crystal length is 7.27 μm, mean width is 1.55 μm, and mean ration of width-to-length is 1: 4.7. The crystals were not always regular in shape and sometimes appeared to have their growth modified by the presence of a helical wall thickening (Fig. 3). By energy dispersive spectroscopy (EDS), these crystals were found to be composed of a calcium compound, most likely calcium oxalate (Fig. 13).

Although single isolated crystals were most commonly found, joined double crystals with U-shaped conjoined end were also observed (Fig. 4). Crystals in groups of two or more were also encountered and had either parallel or perpendicular orientation to each other (Figs. 1,5). Crystals were found in various positions within the tracheary lumen. They were located between either the helical thickenings of the wall material, laying flat on the inner cell wall, or projecting out from the pit membrane (Fig. 1).

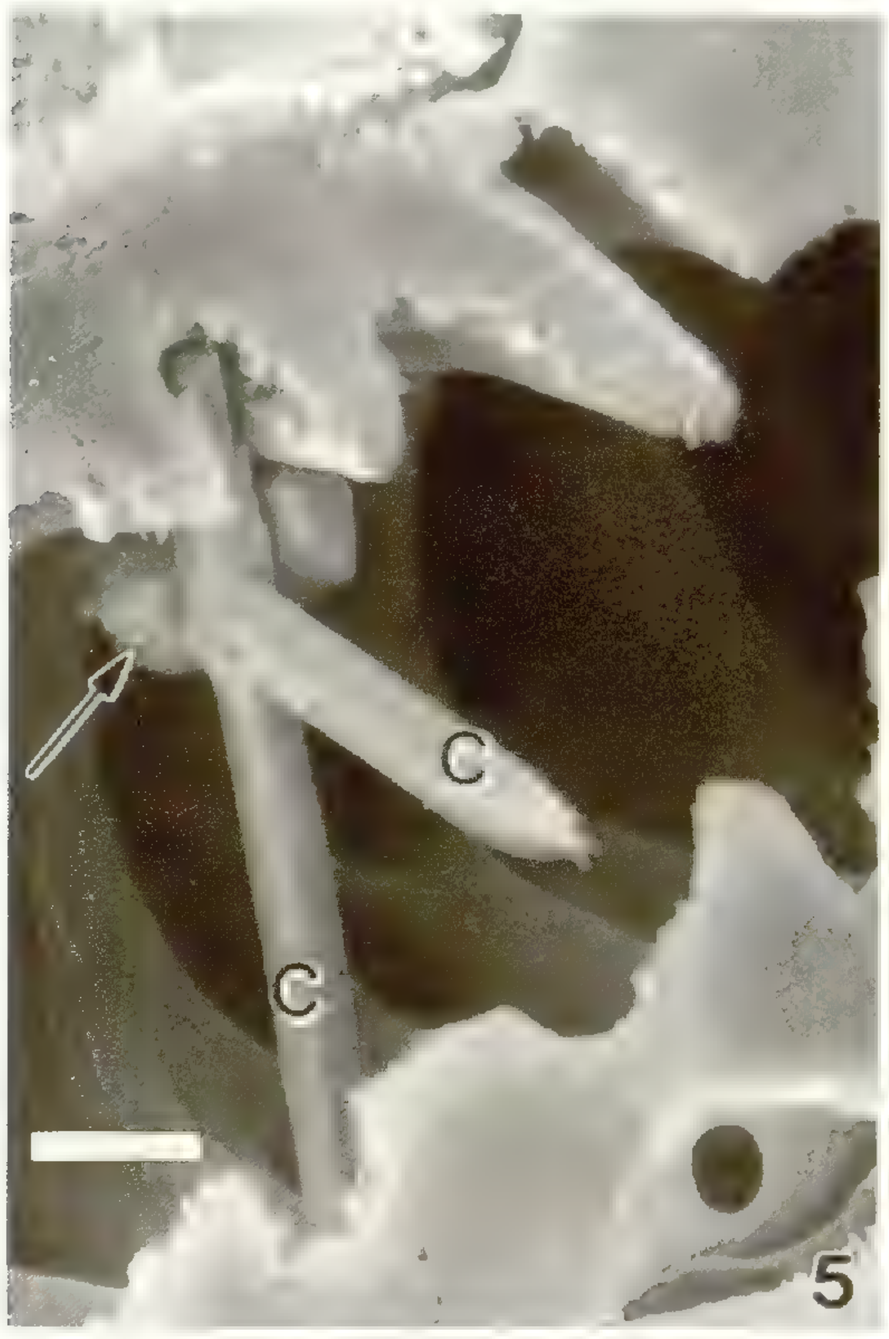
It was clear from some specimens that the crystals were composite structures (Figs. 4, 6–8). In some instances the subunits resembled raphides (Figs. 6,7),

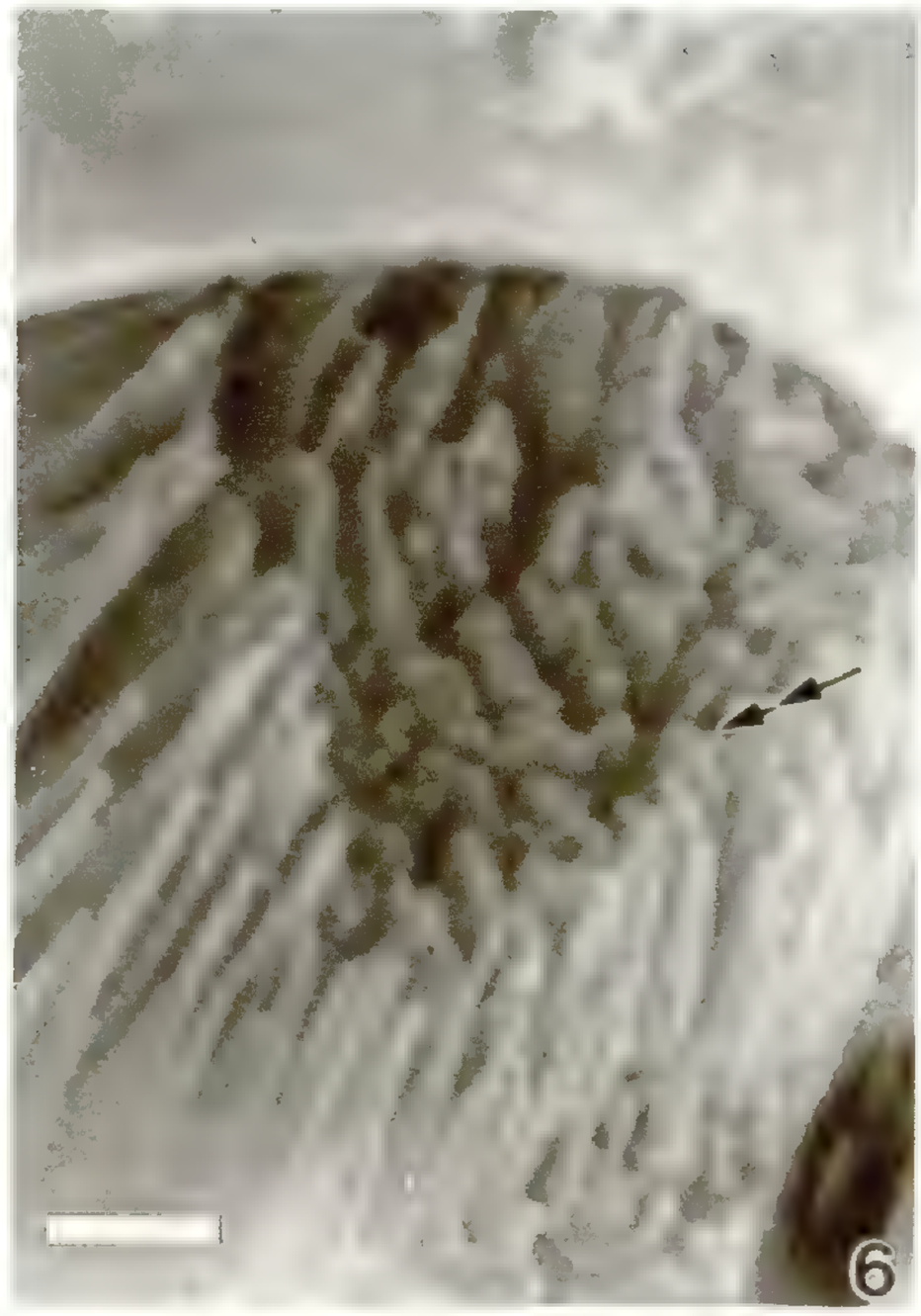


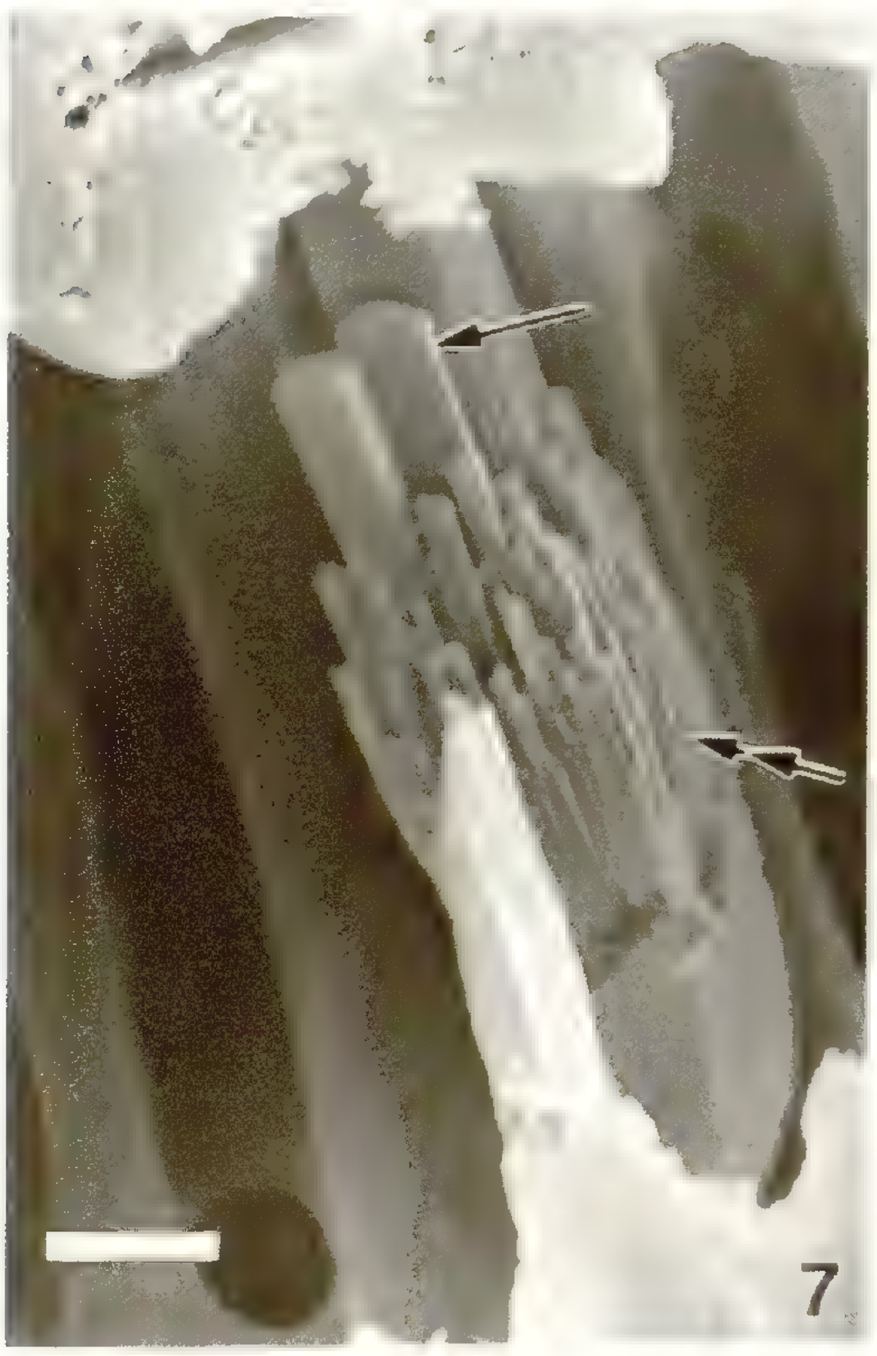


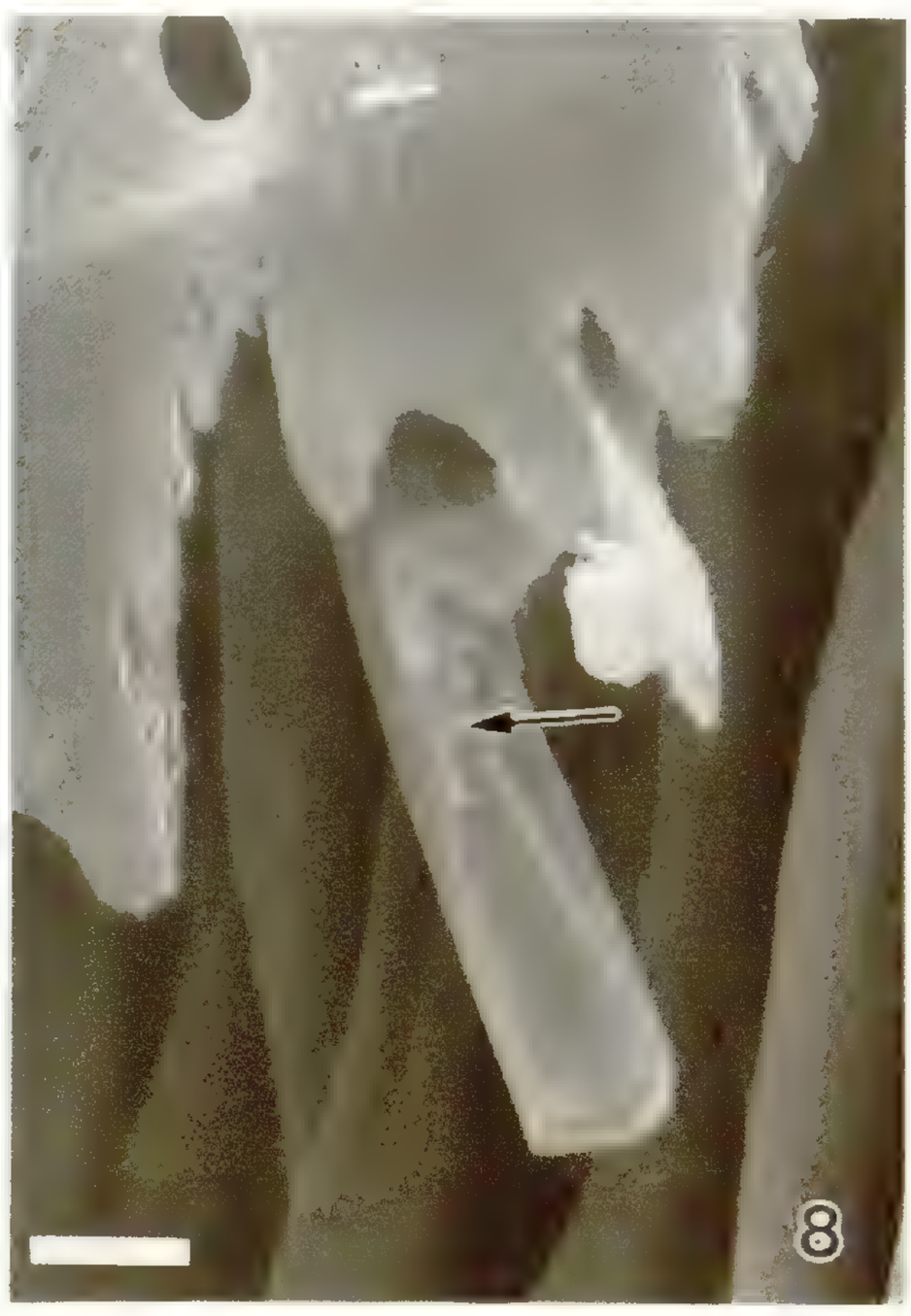


FIGS. 1–4. SEM micrographs of intertracheary pit membranes and crystals. 1) Tracheary lumen with helical wall thickenings (W), crystals (arrow), pit aperture (A), and circular bordered pit membrane (P); scale bar = 5 μm. 2) Intertracheary pit membrane with torus (T) and margo (M). The pit border was removed when the wood was split during preparation; scale bar = 2 μm. 3) Crystal entering a pit aperture (A). Note how the crystal appears to have grown around the helical thickening to the right (double arrow). R = rough end of crystal; scale bar = 2 μm. 4) Double crystal joined at one end (arrow); scale bar = 2 μm.









FIGS. 5–8. SEM micrographs of crystals in which crystals reveal their subunit composition. 5) Multiple crystals with parallel orientation and perpendicular orientation. Note subunits in broken crystal (arrow); scale bar = 5 μm. 6) End view of composite crystal formed by smaller raphide shaped crystals (arrow); scale bar = 500 nm. 7) Composite crystal with styloid (arrow) and raphide crystal (double arrow) shaped subunits; scale bar = 2 μm. 8) Composite crystal with styloid crystal subunits (arrow); scale bar = 2 μm.

whereas in others they resembled small styloid crystals that were fused to form one large crystal (Figs. 7, 8). Both types of subunits appear to integrate into one another (Fig. 7). One shattered example had a hollow center (Fig. 9).

Crystals were observed to traverse the pit aperture (Figs. 3, 10) and contact the pit membrane (Figs. 11, 12). These did not appear to penetrate the pit membrane, but we are uncertain of this point due to the poor preservation of the pit membranes in our samples. Fig. 12 demonstrates a unique occurrence in which a pit membrane is approached by a crystal from either side. Due to the position of the crystal relative to the pit membrane, we were unable to confirm the presence of a torus in each crystal-associated pit membrane; however a torus was present in the samples that exhibited a crystal behind the pit membrane (fig. 11).

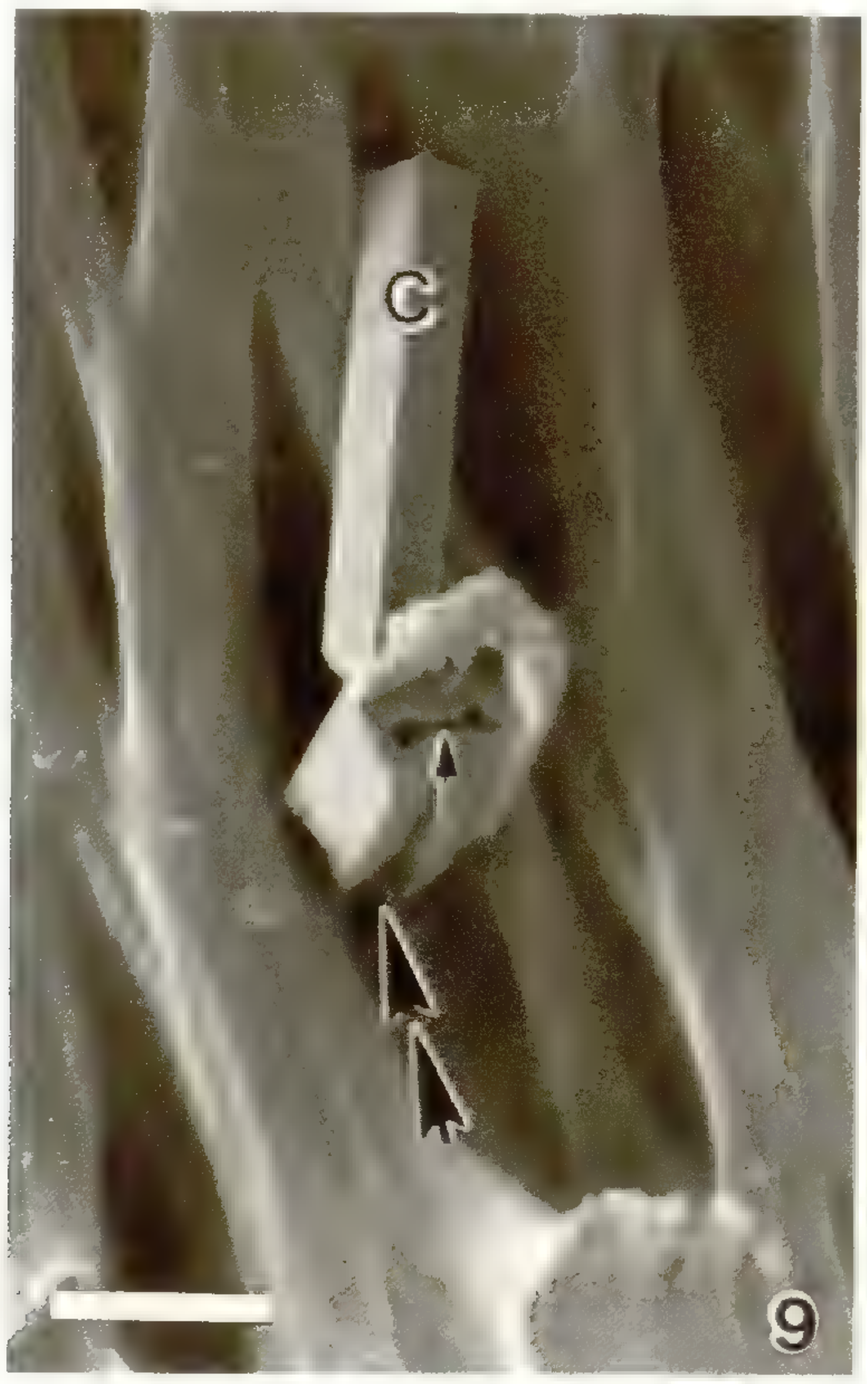
No noticeable chamber or crystal sheath was ever observed in association with a crystal. No evidence of a surrounding membrane was discovered, although the ends of the crystals often were rough (Fig. 3). Efforts to examine crystals with TEM to determine the presence or absence of a chamber or crystal sheath were not successful. Crystals were not observed in either *Botrychium dissectum* or *B. virginianum*.

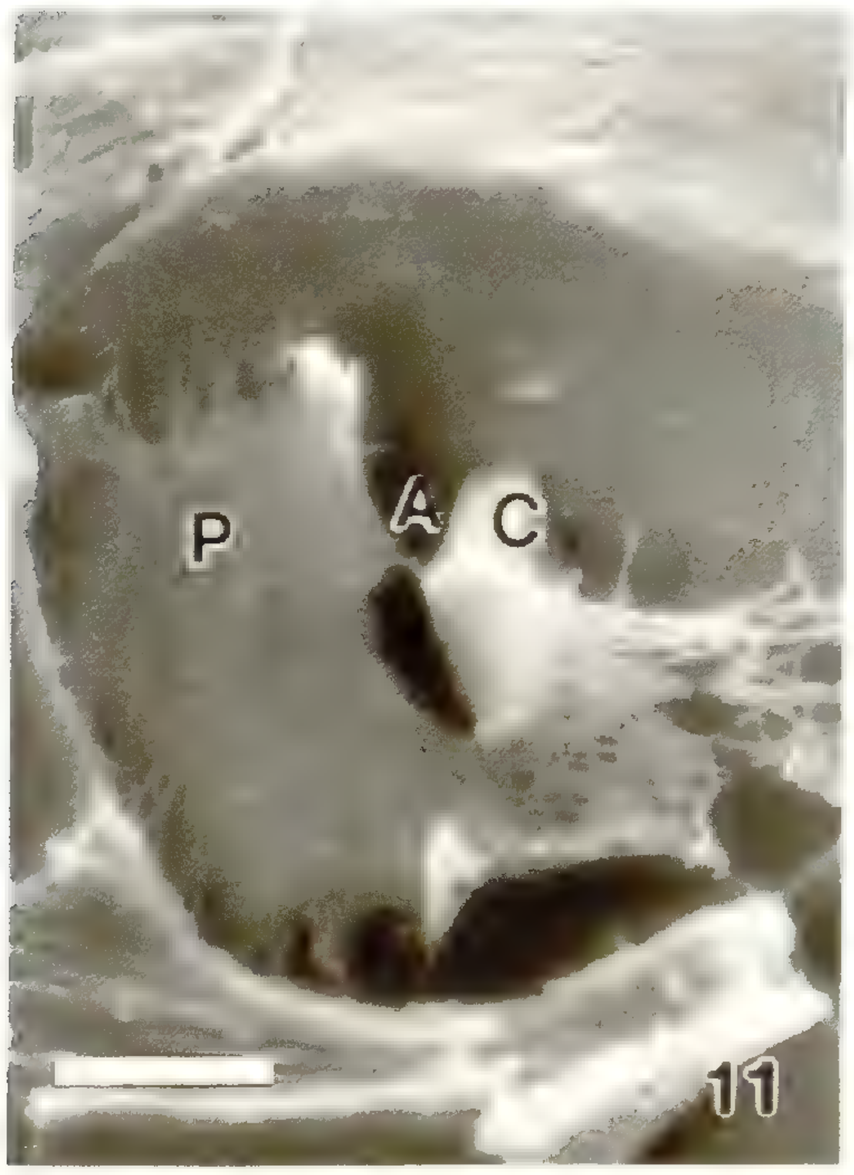
Discussion

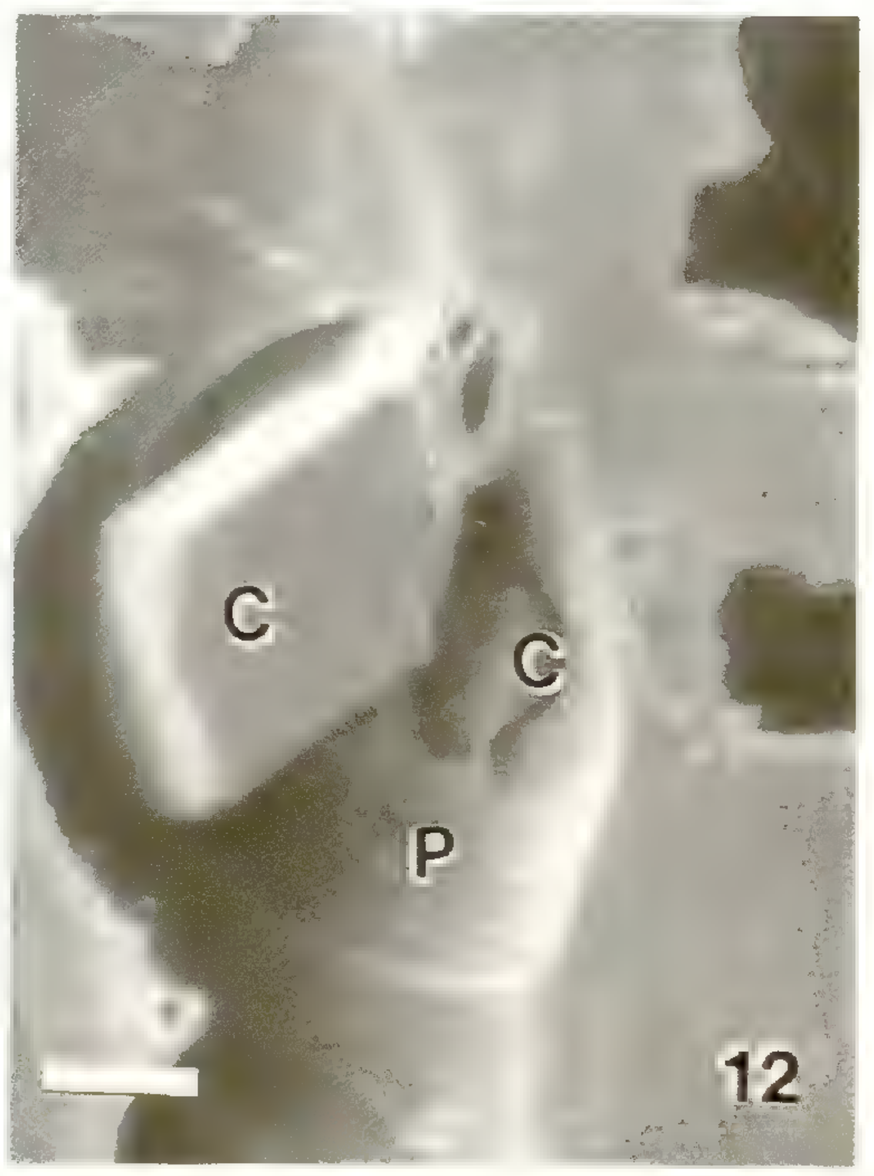
Three major systems of mineralization occur in plants. These include silicification, calcium carbonate crystallization, and calcium oxalate crystallization (Grimson et al., 1982). Calcium oxalate crystals, either in the monohydrate or polyhydrate state, are the most common mineral deposits (Webb and Arnott, 1982). EDS evidence indicates that our crystals contain calcium. The bipyramidal shape of the crystals' ends, and the rectangular columns, suggest that they are crystals of calcium oxalate in the polyhydrate form (Frey-Wyssling, 1981). Usually, acid solubility tests are used to confirm crystal composition in plants (Webb and Arnott, 1982). In addition, the oxalate nature of a calcium crystal can be tested with cupric acetate and ferric sulphate (Deshpande and Vishwakarma, 1992). However, due to the small size and sparse number of crystals found in *Botrychium multifidum*, these tests were not performed.

The location of crystals in tracheid lumens in unusual. Crystals in wood are most frequently found in ray or axial parenchyma cells (Chattaway, 1955, 1956), although they may also be found in septate fibers, vessel tyloses, and even in vascular cambia (Deshpande and Vishwakarma, 1992). In *Polyalthia*, vessels contained a crystalline mass (Scurfield and Mitchell, 1973). In the current study, crystals were isolated within the tracheary lumen, and there was no evidence suggesting attachment to cell walls. Some crystals appeared to be touching, but were not attached to, the pit membrane. Crystals also were found that had no apparent association with a pit membrane. Therefore, it appeared that crystal formation was not directly related to pit membranes.

Crystals in plants often are formed in membrane-bound compartments within the vacuole (Arnott and Pautard, 1970; Franceschi, 1984; Webb *et al.*, 1995). As proposed by Arnott and Pautard (1970), the cell membrane may control

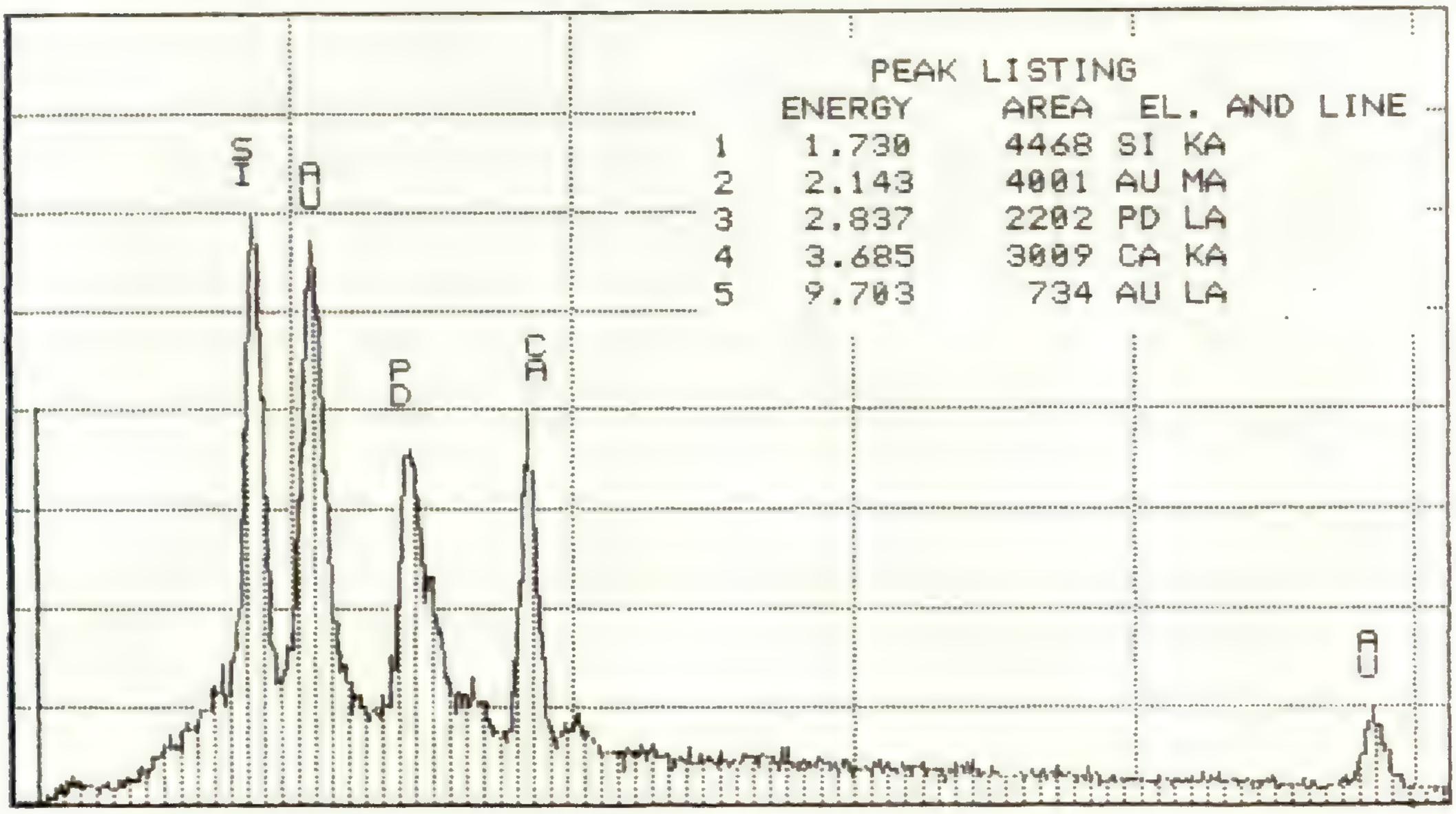






FIGS. 9–12. SEM micrographs of crystals in association with pit area and pit membranes. 9) Fractured crystal with hollow center (arrow). Note the aperture behind the crystal (double arrow); scale bar = 5 μm. 10) Crystal entering a pit aperture. Note shaping of the crystal around helical thickening (arrow); scale bar = 2 μm. 11) Crystal behind pit membrane; scale bar = 2 μm. 12) Pit membrane associated with crystals from contiguous tracheids; A = aperture; C = crystal; P = pit membrane; scale bar = 2 μm.

A. Experimental



B. Control

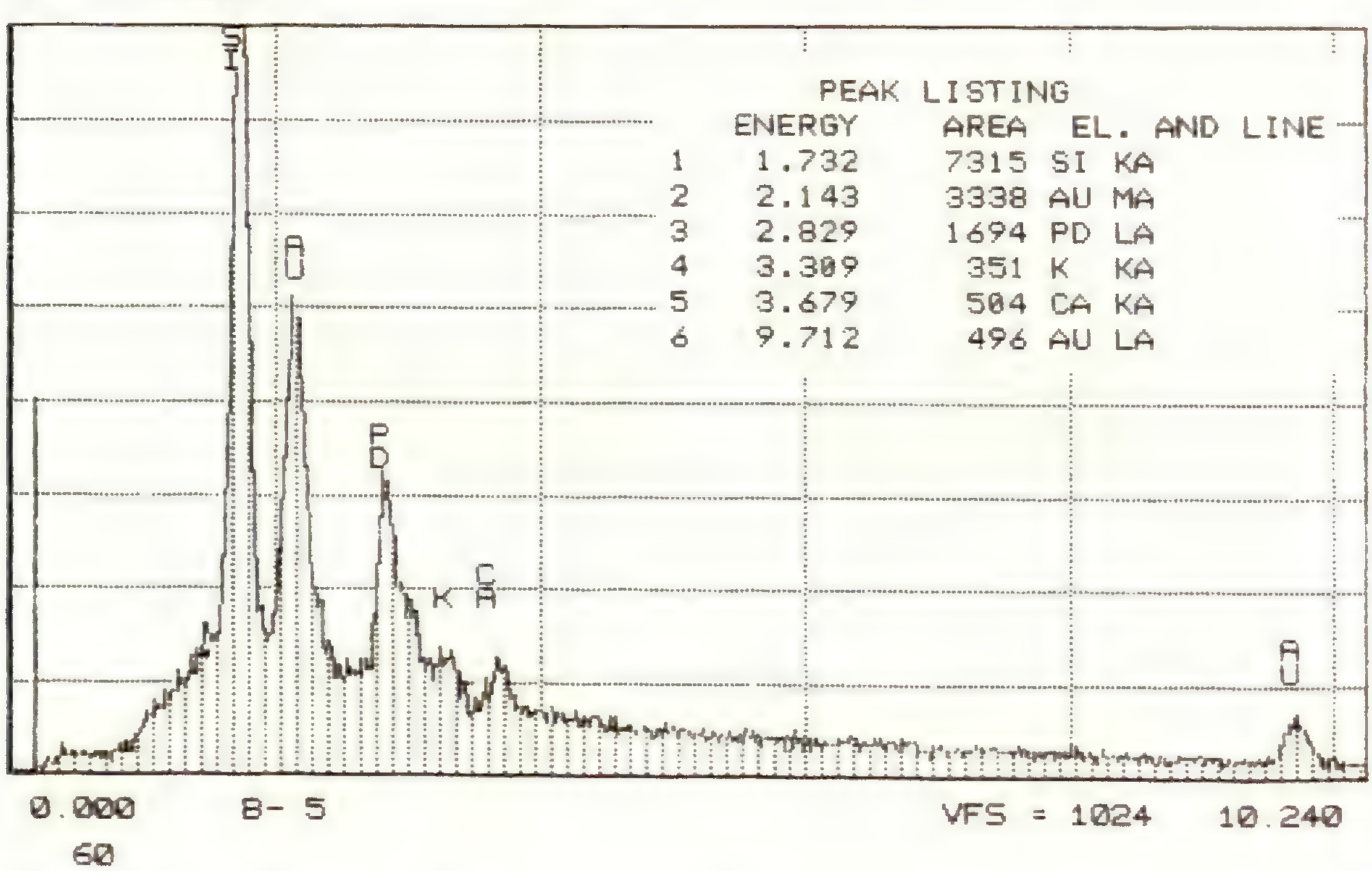


FIG. 13. EDS of tracheid. A. Spectral tracing of crystal within a tracheid. The calcium component of the spectrum is conspicuous and is indicated by the peak labeled CA. B. EDS of tracheid without crystal. Silicon, palladium, and gold are present as background elements (q.v. see preparation procedures in Materials and Methods). Only a small calcium peak is present. The latter probably represents calcium in the middle lamella. AU = gold; CA = calcium; PD = palladium; SI = silicon.

both shape and growth of crystals. There was no direct evidence that crystals of *B. multifidum* were once enclosed in a membrane, but Scurfield and Mitchell (1973) suggest that a rough area on a crystal is indicative of the adhering remnants of membrane. In crystals of *B. multifidum* only the membrane's impression on a crystal would be evident because living portions of the tracheid has undergone autolysis and no membrane remains. If the vacuole with its membrane-covered crystal pressed against either the cell wall or a cell wall thickening as a crystal formed, this contact could explain the shape of these crystals.

Water flow though the xylem could also deposit crystals (no longer enclosed by cytoplasm) randomly throughout a tracheid, including on top of a pit membrane or between wall thickenings. Due to erosion, water flow might also change crystal shape.

Another aspect of crystal development in plant cells in isolation of a crystal by wall material or a suberized sheath after the crystal has formed within a vacuole. This process would, in essence, externalize the crystal (Frank and Jensen, 1970). In *Agave* (Agavaceae), crystals are produced in such extraplasmic compartments (Wattendorff, 1976a, b). Wattendorff (1976b) found that all styloid idioblast of *Agave,* where they did not touch the wall, were surrounded by a suberized sheath. Although crystals of *B. multifidum* are styloids, they appear neither to be associated with a sheath of any sort nor to be isolated by cell wall material.

The reason a cell forms a crystal is not well understood. Crystal formation may represent a crystallization of waste material or storage of minerals (Deshpande and Vishwakarma, 1992). Crystal formation also may be associated with ionic balance, and therefore, the formation of a crystal could be a form of osmoregulation (Franceschi and Horner, 1980). Franceschi and Horner (1979) correlated the amount of calcium in the growth medium and the number of crystals formed in *Psychotria* L. (Rubiaceae) callus. Lane (1994) has suggested that calcium oxalate crystals may promote the polymerization of lignin which of course, would be occurring in the developing tracheids of B. multifidum.

It is evident at times that crystal formation in plants is under genetic control (Frey-Wyssling, 1981; Webb, 1999); however, genetic control of the formation of all crystals has not been proven. The cell in which a crystal is produced undergoes many changes at macro, micro, and ultrastructural levels, as well as, changes in cell chemistry. These changes, documented in other plant taxa during crystal formation, make it unlikely that crystal formation could be simply the result of precipitation or crystallization (Franceschi and Horner, 1980), although crystal formation may represent crystallization of waste material or storage of minerals (Deshpande and Vishwakarma, 1992). Deshpande and Vishwakarma (1992) also identified seasonal fluctuation in crystal formation after the cessation of cambial activity. Gourley and Grime (1994) described crystals that were more commonly found in the late wood of *Acacia* Mill.(Fabaceae). The availability of water was also determined to be a factor in crystal formation (Gourley and Grime, 1994).

It was impossible to determine for certain whether crystals in the tracheids

of *B. multifidum* formed before or after cell death. Perhaps due to greater water flow resistance occurring at the pit membrane, there would have been a greater chance for calcium precipitation in the pit area rather than in the tracheary lumen. If this were the case, crystals could at the pit membrane form after the death of a tracheid. However, the rough ends observed on some crystals suggest they may have been enclosed at one time by a membrane. Additionally, the crystals appear to conform to the shape of the pit aperture or cell wall thickenings and do not appear to have been randomly distributed by water flow. Perhaps the best explanation of where these crystals develop is in membrane compartment within vacuoles of living tracheids. The enlargement of a crystal in a plane perpendicular to the cell's axis would result in its abutting a wall or pit membrane, thus influencing crystal shape. Crystals that elongated parallel to a cell's axis would not encounter these boundaries and would not be shaped by them. Crystals that were not pressed into a cell wall or pit membrane also would not develop this shaping and might settle between wall thickenings, or after cell death, move with the xylem water stream. However, the positions of crystals in Figures 3, 10, and 12 with respect to the pit membrane suggest that crystal position is not the result of water flow.

Crystal formation has also been associated with the products of fungal metabolism within the plant cells (Scurfield and Mitchell, 1973). In our study, no fungal hyphae were found near any of the crystals. Therefore, this possibility in *B. multifidum* seems unlikely.

If crystal manufacture is under genetic control, what advantage does the cell gain from its production? This is an especially intriguing question with regards to *Botrychium* as crystal production would be occurring in cells about to die. Lane (1994) has suggested that calcium oxalate crystals play a role in lignin polymerization and perhaps this may be true in these lignified tracheids. However, the lack of crystals in other *Botrychium* species indicates that this would be true only under certain environmental conditions. As previously mentioned, some authors believe that crystals represent the storage of calcium that could be either reserve calcium or waste calcium (Deshpande and Vishwakarma, 1992; Webb, 1999). Storage of needed calcium in a near-death cell would be unlikely. However, these crystals may have been produced by a cell for ionic balance or osmoregulation. Ionic balance and osmoregulation are critical for immature cells (Franceschi and Horner, 1980). If a plant were growing on soil with high nitrate levels, assimilation of this compound would increase cell pH, and oxalic acid might be produced to counter this effect. The oxalate anion could then react with calcium to form a crystal that would remove the excess anion from cell sap (Franceschi and Horner, 1980).

Another explanation could be protection from herbivores, although crystal production in a leaf cell would be more plausible for defense purposes. The crystals in *B. multifidum* are too small and too few in number for this type of protection. Fire protection was listed as an explanation by Gourley and Grime (1994) for crystals in *Acacia*, but again this is unlikely for a rhizome.

Based on our data the best explanation for crystal formation in the xylem of *B. multifidum* is that crystals are the result of excess calcium precipitation,

which could represent either waste, storage, or osmoregulation in the plant. Because the crystals are located in dead cells, active resolubilization by a cell would be unlikely; however, if crystals were dissolved by water flow in the xylem, their minerals could be carried in the transpiration stream. Deshpande and Vishwakarma (1992) have suggested that formation of calcium crystals may be a reversible process in some tissues. Therefore, these crystals do not necessarily represent a calcium loss for the plant.

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A New Filmy Fern from the Dominican Republic

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ABSTRACT.—A new species of *Hymenophyllum* subg. *Hymenophyllum* with entire involucral valves is described from the Dominican Republic on the island of Hispaniola.

During the prepartion of a revision of the filmy ferns (Hymenophyllaceae) for the Flora of the Greater Antilles project, a peculiar species was discovered among the undetermined specimens in the Gray Herbarium.

The taxon belongs to *Hymenophyllum* subg. *Hymenophyllum,* following Morton (1968, pp. 162–164), a subgenus represented by only two species in the Antilles. *Hymenophyllum tunbrigense* (L.) Smith is known only from Jamaica and Hispaniola (Proctor 1985, p. 90), whereas *H. fucoides* (Sw.) Sw., is more widely distributed (Proctor, 1985, p. 92; 1989, p. 58). The members of this subgenus are characterized by having toothed segment margins, and most have sinuous to toothed involucral valves as well. The new species is described as

Hymenophyllum integrivalvatum C. Sánchez *sp. nov.* Fig. 1

Ab speciebus aliis antillanis subgeneris *Hymenophylli* valvis integris, stipitibus brevissimis, segmentis pinnarum paucis (1 vel 2), necnon laminis glaberrimis diversa.

TYPE—**Dominican Republic**: Pcia. La Vega: Near the pyramid ca. 13 km from Valle Nuevo on the road to San José de Ocoa, ca. 2500 m elev., 22 August 1957, *Gastony, Jones & Norris 740* (GH; isotype US).

Rhizomes creeping, filiform, 0.1–0.3 mm in diam., clothed with deciduous, brownish, pluricellular trichomes, with a few conspicuous, straight roots ca. 5 mm distant. Fronds small, erect, determinate, approximate, 1.15–2.1 cm long.; stipes 0.1–0.3 mm long., 0.2 mm in diam., very narrowly alate throughout, dark brown, glabrous or with a few brownish, often 2-celled trichomes; laminae narrowly ovate, lanceolate or oblong 1.4–1.8 cm long, × 0.8–1 cm wide, pinnate-pinnatifid; rachises notably flexuous, narrowly and evenly alate, the alae less than 0.1 mm wide, dark brown, glabrous; pinnae 5–8 pairs, spreading to ascending, mostly with 2 acroscopic segments; segments narrowly elliptic, oblong, or linear-oblong, 1.2–1.8 mm wide, glabrous, the margins distantly toothed, the teeth usually more distant than their length and ascending, the midvein dark brown, the lamina tissue olivaceous-green when dry; sori conspicuous in size in comparison with the length of the lamina, borne at the lamina apex or in the distal half, subaxillary on the acroscopic side of the

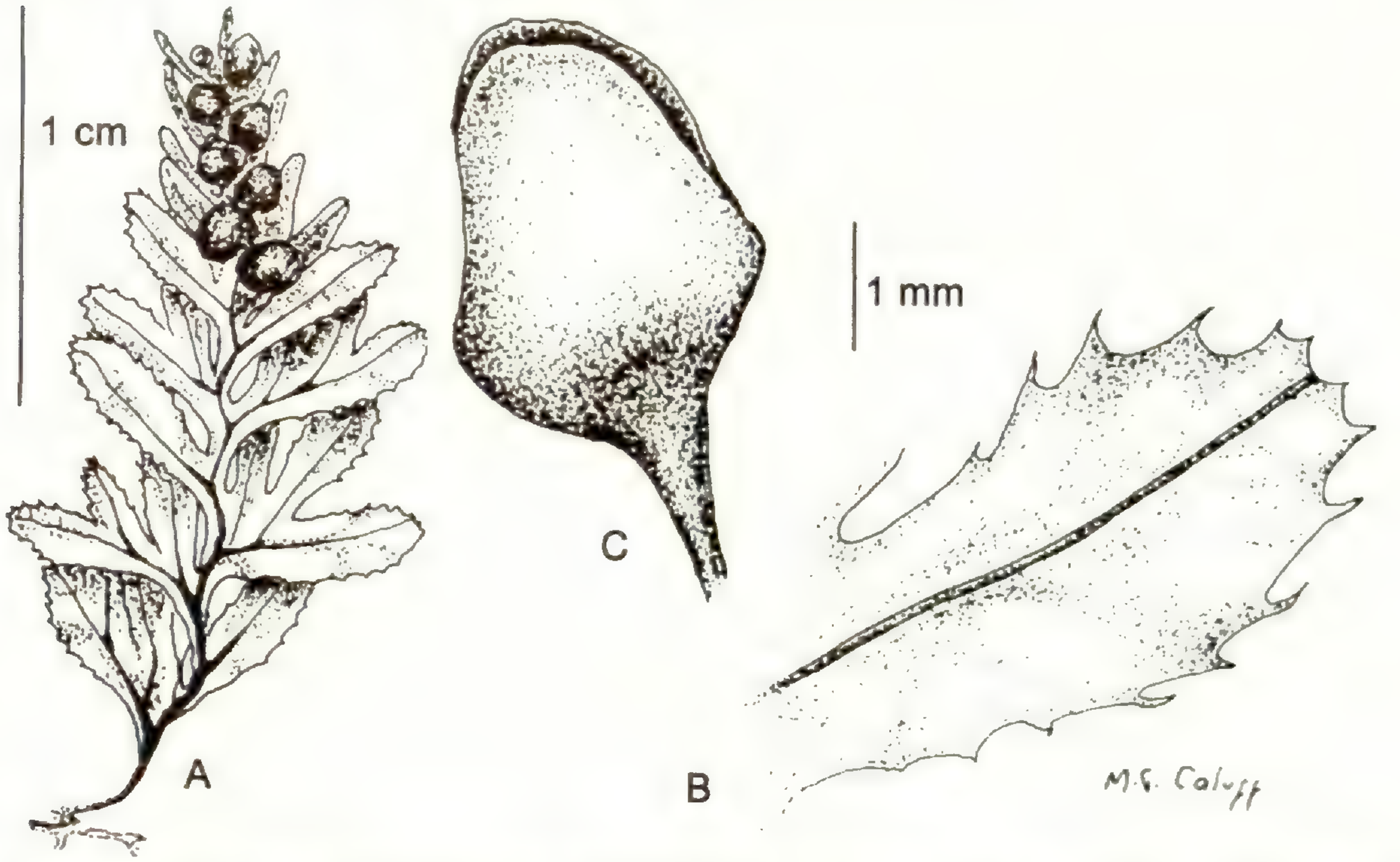

FIG. 1. Holotype of *Hymenophyllum integrivalvatum* (GH). A. Habit of plant. B. Detail of an ultimate segment. C. Detail of a sorus.

pinnae; involucres 1.6–2.2 mm long, 1.4 mm wide, broadly elliptic or broadly ovate, bivalvate, the valves wider than the sterile segments, the margin entire, the filiform receptacle included.

DISTRIBUTION.—Endemic to Hispaniola (Dominican Republic), known only from the type collection.

HABITAT.—Epipetric in very moist burned and timbered pinelands, forming thick mats on rocks along streams in very moist ravines, according to the information on the label.

The new species is most closely related to *H. tunbrigense* (L.) J. E. Smith, which is also known from a few collections from Hispaniola. *Hymenophyllum tunbrigense* differs in having larger fronds, straight rachises with wider alae, more divided pinnae, narrower segments, and sinuous involucres. The entire involucral valves, very small fronds, and the absence of trichomes on the remainder of the lamina separate the new species from all other Antillean species of subg. *Hymenophyllum*.

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Adiantum argutum, an Unrecognized Species of the *A. latifolium* Group

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ABSTRACT.—The present paper distinguishes *A. argutum*, an unrecognized but widespread species from South America, from the related *A. latifolium*, and designates a lectotype for *A. argutum*.

Several pinnate or bipinnate *Adiantum* species have an indument like that of the *A. serratodentatum* group, but differ in having fewer, larger, less dimidiate pinnules and thin, very long-creeping rhizomes. Among the species of this group are *A. argutum* Splitg., *A. incertum* Lindm., which is based on *Lindman Regnell Exped. I. A2083* (S not seen; isotypes B, GH) from Paraguay, the widespread *A. latifolium* Lam., and *A. viviesii* Proctor, which is based on *Proctor 41389* (US; isotypes IJ, SJ) from Puerto Rico. *Adiantum glaziovii* Baker, which is based on *Glaziou 13345* (K, isotype US) from Rio de Janeiro, Brazil, is a synonym of *A. latifolium*. The early, unplaced name *A. elatum* Desv., which is based on a Brazilian specimen from the Herb. Dombey (P-Herb. Juss. Cat. 1421 not seen Morton photo 3153) will likely displace one of the later named species found in Brazil. The specimen was said by Morton to have almost glabrous segments; it needs to be examined critically.

Adiantum argutum Splitgerber, Tijdschr. voor Natuurl. Gesch. en Physiol. [Leiden] 7: 427. 1840. Figs. 1, 2.

Lectotype (chosen here): "in sylvis montosis Surin. prope Bleauwe Berg," Surinam, May 1838, *Splitgerber 891* (L not seen, photo US). Other syntype: *idem, id., Splitgerber 290* (L not seen, photo US).

Adiantum fovearum Raddi var. *reductum* Jenm. Ferns Br. W. Ind. & Guiana 87. 1899. TYPE: Not clearly stated, but presumably Guyana, *Jenman* (NY? not seen).

Splitgerber (1840) described *A. argutum* based on material he collected in Surinam. The author did not mention any specimens in the protologue, only the locality. Original materials were found by Morton at Leiden (Morton photos 193 and 194, both US), and they may be considered the syntypes of Splitgerber's taxon. The lectotype selected here (*Splitberger 891*) has an original label, handwritten by the author with the same information he published with the

FIG. 1. Range of *Adiantum argutum* Splitg.

original description. The photograph of the other syntype (*Splitgerber 290*) resembles *A. latifolium* Lam., although we can not place it there with certainty.

According to Splitgerber (1840), *A. argutum* has a long-creeping rhizome; the laminae are lustrous adaxially and have 3 or 4 pinna pairs, acuminate pinnules, a subrhombic terminal pinnule, reduced and flabellate basal pinnules, sparse, minute setiform scales abaxially, and oblong sori. In fact, these characters distinguish this species from all others closely related to it. Other important features to recognize *A. argutum* are the distant fronds and the idioblasts on the abaxial surface of the pinnules.

Unfortunately, over the years, the Splitgerber species was included within the concept of *A. latifolium* by Vareschi (1969, p. 734), Kramer (1978, p. 91), Tryon and Stolze (1989, p. 66), and Smith (1995, p. 259). In other cases, the

name was synonymized under this species, by Posthumus (1928, p. 105), Lellinger (1989, p. 148), and Cremers and Hoff (1990, p. 19).

Adiantum argutum demonstrates its close affinity to *A. latifolium* mainly in its slender, long-creeping rhizome, 2-pinnate fronds, and stipe and rachis covered by deltate to lanceolate scales with a pectinate base. However, *A. latifolium* differs by its smaller, obtuse to subacute pinnules with a roundish apex that are abaxially glaucous, glabrous, and without idioblasts.

Adiantum incertum Lindm. differs from *A. argutum* and *A. latifolium* in having the scales on the abaxial surface of the pinnules hairlike and with a few basal processes, rather than having such scales with a pectinate base or totally lacking scales. In addition, it is a species restricted to Paraguay and extra-Amazonian Brazil: Goiás (Maurilândia, Rio dos Bois, *Hatschbach 34271*, MBM, MO, NY, UC), Mato Grosso (Santa Terezinha, 21 km SW of Portal da Amazônia, *Thomas et al. 4334*, NY, US), São Paulo (São Carlos, 9 km NNE of the BR Station at Santa Eudóxia, *Eiten & Eiten 3488*, US), and Paraná (Foz do Iguaçu, Parque Nacional das Cataratas, *Hatschbach 23171*, HB, MBM, MO, UC, UPCB).

Adiantum obliquum Willd., although its fronds look much like those of *A. argutum*, can be distinguished by its short-creeping rhizomes, approximate and usually 1-pinnate fronds, and pinnules with conspicuous idioblasts on both surfaces. It may be more related to *A. lucidum* Cav. and perhaps to *A. petiolatum* Desv., with which it hybridizes. These three species may form a separate group.

Adiantum argutum has a more restricted area of distribution than its closest relative *A. latifolium*. It occurs in northern South America (Colombia to French Guiana) and in the Amazonian regions of Peru, Bolivia, and Brazil. It grows in primary and secondary forests, on dark red lateritic clay soils, from 50 to 1000 m elevation.

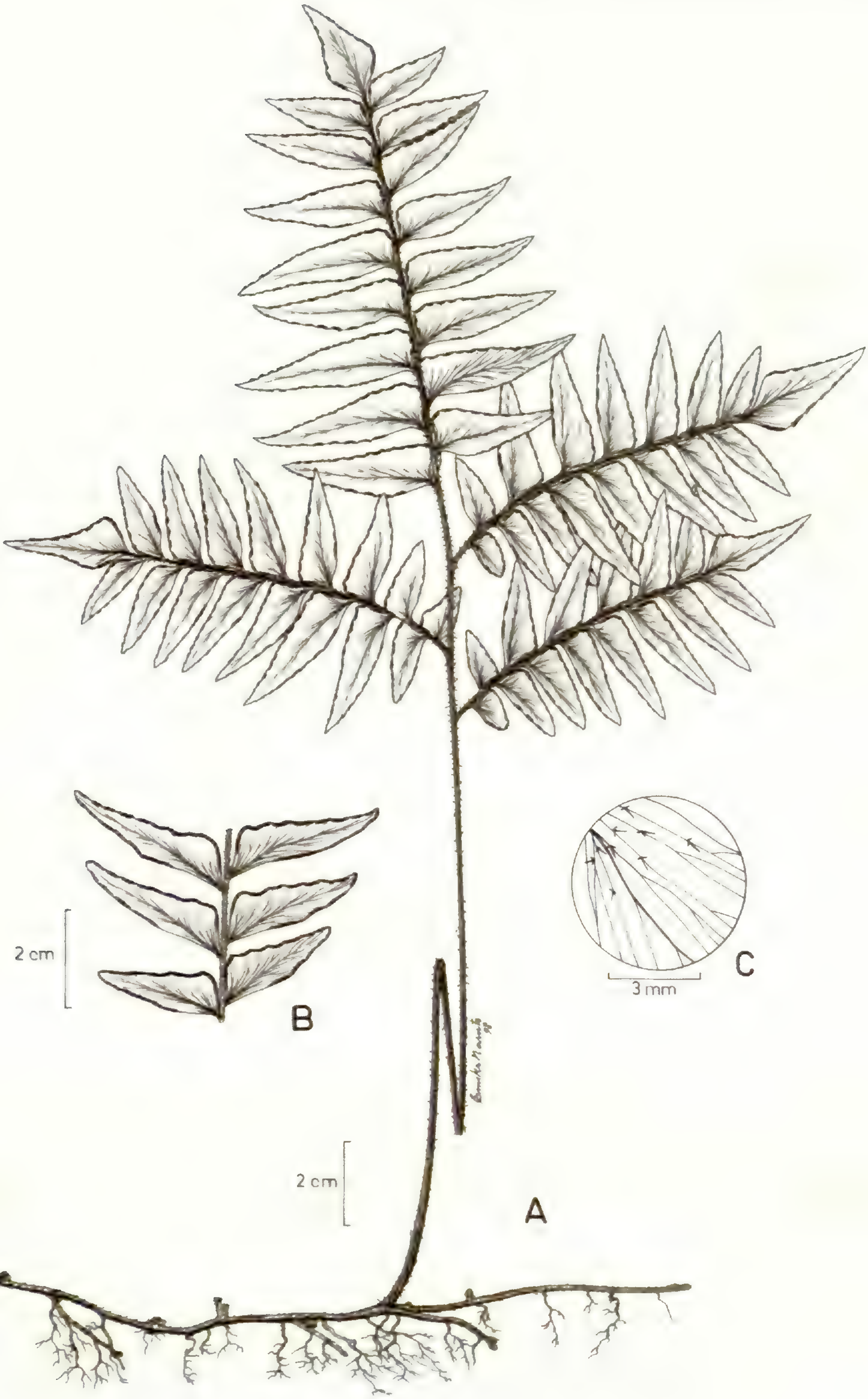
Representative specimens of *A. argutum* studied:

COLOMBIA: **Meta**: Sierra de La Macarena, Caño Entrada, *Philipson & Idrobo 1748* (US); Villavicencio, *Pennell 1607* (GH). **Boyacá**: Los Llanos, *Haught 2833* and *2844* (both GH). **Vichada:** San José de Ocumé: near Río Vichada at Botomi, ca. 14 km NW, *Hermann 11107* (US); NE de Pto. Inírida, 3°58′N, 67°50′W, *Churchill et al. 17748* (NY).

VENEZUELA: **Bolívar**: La Tomasa, *Williams 1295* (US); Río Paragua, Isla El Casabe, *Killip 37301* (US); Salto Alta, Alto Orinoco, *Croizat 486* (NY); Dtto. Sifontes, Concesión Minera Oro Uno, 7 km NW of la Clarita, 6°13′N, 61°27′W, *Aymard et al. 3976* (NY); Sierra Imataca betw Río La Reforma and Puerto Rico, N of El Palmar, *Steyermark 88012* (US). **Amazonas**: Around the margin of the Río Orinoco above Tamatama, *Williams 15199* (GH); Cuenca del Río Manapiare, 5°5′N, 66°03′W, *Huber 435* (NY). **Delta Amacuro**: Río Cuyubini, Cerro de la Paloma, *Steyermark 87649* (NY). **Mérida**: Near border Río Grande de Toro, 61°44′W, 80°4′N, *Breteler 3781* (US).

TRINIDAD: *Fendler 2* (NY).

GUYANA: **Cuyuni-Mazaruni**: 8 km N of Bartica on W bank of Essequibo River, 06°29′N, 58°38′W, *Henkel & Chin 297* (US). **U. Takutu-U. Essequibo**:


2 cm
B
3 mm
C
2 cm
A

Rupununi area, Surama Village, 04°08′N, 59°04′W, *Acevedo et al. P3297* (US); Marudi River, 02°11′N, 59°11′W, *Henkel et al. 2902* (NY), *3032* (US); Kuyuwini River, 02°11′N, 59°11′W, *Henkel et al. 3022* (US); NW Kanuku Mts., 3°21′N, 59°30′W, *Hoffman & Foster 3510* (US); Rupununi River, *Jansen-Jacobs et al. 4207* (US). **Potaro-Siparuni:** On 0.5 km island in Essequibo River, 1 km S of Fairview, 4°40′N, 58°40′W, *McDowell 3371* (US); Iwokrama Mts., Annai-Karupukari Rd., 04°19′N, 58°51′W, *Hoffman et al. 1409* (US); River Isherton, 2°20′N, *Smith 2432* (GH, NY). **Barima-Waini**: Head of Barima River, Ayamba Falls, 4.5 mi W of Eclipse Falls, ca. 10 km W of Arakaka, 7°39′N, 60°09′, *Pipoly & Lall 8200* (NY); Head of Barima River, NW of Kariako River, 7°30′N, 60°35′W, *McDowell 4393* (NY); Labbakaka Creek, Tiger Creek, *Sandwith 1209* (K, NY).

SURINAM: **Haut Litany:** Basin du Litany, 2°31′N, 54°45′W, *Granville et al. 12040* (US). **Nickerie:** Area of Kabalebo Dam project, *Lindeman & Roon 884* (US); Area of Kabalebo Dam project, 4°–5°N, 57°30′–58°W, *Lindeman et al. 165* and *343* (both NY); Sectie O, along railroad, vic. Km. 70, *Maguire & Stahel 23605* (GH, NY). **Brokopondo:** 2.4 km S of village Gansee, *Donselaar 1189* and *1276* (both GH); Zuid River, 3°10′–3°20′N, 56°29′–56°49′W, Kayser Airstrip, 45 km above the confluence with Lucie River, *Irwin et al. 57697* (NY).

FRENCH GUIANA: Cayenne, Inini River, 3°28′N, 52°36′30″W, *Cremers et al. 8781* (US); Camp Eugene, Basin du Sinnamary, 4°51′S, 53°4′W, *Cremers & Granville 13727* (NY); Gobaya Soula, Basin du Maroni, 53°58′W, 3°37′S, *Cremers et al. 10125* (US); Saoul, 3°37′N, 53°12′W and vicinity, Route de Bélizon, N of Eaux Claires, *Heald & Yahr 56* and *65* (both NY); Comté., degrad auprés de Crique Martineau, *Oldeman I426* (NY); Mt. Balbao, Secteur Sud, 3°35′N, 53°20′W, *Granville et al. 8958* (NY).

PERU: **Madre de Dios**: Near the confluence of Río Tambopata and Río La Torre, 39 km SW of Puerto Maldonado, 12°50′S, 69°20′W, *Smith & Condor 1114* (US) and *1363* (NY); Tambopata, *Vargas 18577* (GH); Tambopata, vic of Moho towards Piedra Redonda, at the Bolivian frontier, 12°30′S, 69°40′W, *Nuñez et al. 9695* (GH, NY); Tambopata, SSW of Pto. Maldonado at the confluence of the R. La Torre and the R. Tambopata (SE bank), Tambopata Nature Reserve, 12°49′S, 69°17′W, *Barbour 4763* (NY), *López 4585* (GH).

BOLIVIA: **Beni**: Pcia. Ballivian: Río Colorado, Collegio Técnico Agropecuario de Río Colorado, 15°00′S, 67°10′W, *Fay & Fay 2105, 2640, 2652, 2654, 2681* (all US); 18.4 Km E of Riberalta, then 1 km NE on old road to Cachuela Esperanza, 11°05′S, 65°50′W, *Solomon 7804* (NY); Isla Capanario, 50 m from the San Borja–San Ignacio de Moxos road, 212 km from Campoamento Totaizal, *Rolleri 140* (NY); Pcia. Moxos: Chimanes Forest, 15°10′S, 66°37′W, *Fay & Fay 2794* (US). **Sta. Cruz**: Bella Vista, Río Blanco, *Scolnik & Luti 681* (US); Pcia. Ichilo: Old meander loop of the Río Ichilo, 1–1.5 km SW of the Buena Vista–Villa Tunari Hwy., 17°18′S, 64°12′W, *Nee & Moran 45225* (NY). **Pando:** Nicolas Suarez, SW of Cobija on the Río Naraueda, 11°08′S, 69°08′W, *Sperling*

←

FIG. 2. *Adiantum argutum* Splitg. Fig. 2A. Habit. Fig. 2B. Abaxial surface of some pinnules. Fig. 2C. Abaxial surface of a pinnule showing the scales.

& King 6475 (GH, NY, UEC); Ca. 20 km from Cobija towards Castro Eriña, *Casas & Sussana 8123* (NY); W bank of the R. Madeira betw Cachoeiras Madeira and Misericórdia, *Prance et al. 6612* (NY). **La Paz**: Pcia. Iturralde, Siete Cielos, R. Manupare, 12°27′S, 67°37′W, *Solomon 16947* (NY).

BRAZIL: **Amapá:** Serra do Navio, bank of the Rio Amapari, *Emmerich & Andrade 745* (HB, R). **Roraima**: Posto Mucajaí, Rio Mucajaí, vic of Mucajaí airstrip, *Prance et al. 10991* (GH, R, UC). **Pará**: Lageira, airstrip on Rio Maicurú, 0°55′S, 54°26′W, *Strudwick et al. 3088* (NY), *3129* (MG, NY, US), *3580* (US); Curuá S.A., near Alenquer, Santarém, *Piggott 2547* (K, NY); Breu Branco, ca. 40 km S of Tucuruí, 4°03′S, 49°40′W, *Daly et al. 1376* (GH, MO, NY, US); Serra dos Carajás, Serra Norte, ca. 15 km W of AMZA Exploration Camp., 6°S, 50°15′W, *Berg & Henderson BG472* (GH, NY, UC, UEC, US); Serra dos Carajás, 6°4′S, 50°8′W, *Secco 286* (GH, K, MG, NY, SPF); BR-163, Cuiabá–Santarém Highway, km 885.5, *Prance et al. P25171* (MG, NY, UC, US); Parque Indígena do Tumucumaque, Rio Parú de Oeste, Missão Tiriyo, 2°20′N, 55°45′W, *Cavalcante 2401* (K, MG, NY, US); Conceição do Araguaia range of low hills ca. 20 km W of Redenção, near São João and Troncamento Santa Teresa, 8°03′S, 50°10′W, *Plowman et al. 8635* (GH, NY), *8757* (GH, NY, US); Rio Xingu, *Balée 2398* (NY); Confluência com Rio Pardo, *Vasconcelos et al. 260a* (NY); Rio Itacaiunas, affluent of the Rio Tocantins, Serra Buritirama, 50°15′W, 5°30′S, *Pires 12427* (NY); Rio Cuminá, *Ducke (Hb. Mus. Goeldi 8885, 15163)* (both HB, MG). **Amazonas**: 1–5 km road Boca do Acre to Rio Branco, *Prance et al. 2533* (GH, MG, NY, R, US); Vic. of Tototobí, Basin of the Rio Demeni, *Prance et al. 10208* (NY, UC, US); Rio Curuquete, Providencia, *Prance et al. 14632* (NY, UC); São Paulo de Olivença, 30 km above the mouth of the Rio Coti, *Prance et al. 14444* (B, NY); Vic. of Macujaí airstrip, *Prance et al. 10991* (MG, NY, UC); Borba, 4°02′S, 59°06′W, W side of the Rio Cunamã, *Hill et al. 12868* (MO, NY). **Rondônia**: Mineração Campo Novo, BR-421, a 2 km a Oeste da Mineração Campo Novo, 10°35′5″S, 63°37′W, *Vieira et al. 517* (NY, US); Basin of Rio Madeira, Trail north of Rio Madeira from 2 km, below confluence of Rio Abunã, *Prance et al. 8345* (K, NY, UC, US). **Acre**: Palácio de Castro, fazenda Mococa, ramal no km 120 da rod. Rio Branco–Pôrto Velho, *Santos et al. 122* (MG, NY, US); 9 km from Rio Branco on Rio Branco–Pôrto Acre road at cut-off for Colônia Cinco Mil, *Lowrie et al. 650* (MG, NY, R, US); Sena Madureira, Bacia do Rio Purus, varação para o Seringal Fonte Boa, 10°07′S, 69°13′W, *Silveira et al. 668* (MO, NY); Xapuri, Seringal Cachoeira, 35 km SE of Xapuri, *Pinard 809* (NY), *Kainer 126* (NY). **Mato Grosso**: Colider, Comunidade São Francisco, *Salino 284* (GH); Serra Ricardo Franco, 15°S, 60°W, *Windisch 1503* (HRCB); Rio Peixoto de Azevedo, Faz. São José (Cachimbo), *Bokermann 6747* (UEC); Santa Terezinha, BR-158, Vila Confresa, pr. ao aeroporto da Fazenda Confesa, 10°35′S, 5°35′W, *Windisch 5987* (UC).

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Polypodium vulgare Plants Sporulate Continuously in a Non-Seasonal Glasshouse Environment

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ABSTRACT.—In their natural environments pteridophytes usually have regular sporing periods, the onset of which is triggered by the interaction of climatic and nutritional factors. Little, however, is known about what changes there may be in the sporing behaviour of a fern when it is transferred from its natural habitat to an artificial environment, such as a glasshouse. We recorded sporing behaviour in relation to vegetative growth in two genetically matched populations of *Polypodium vulgare*. One population was placed in a controlled-climate glasshouse, the other was left outside. The recruitment of new fronds was significantly higher in the indoor population than in the outdoor population. The indoor population also maintained a high proportion of actively sporing fronds throughout the winter. There was no net recruitment of new fronds in the outdoor population during the winter and early spring. Some elements of the glasshouse environment, probably the enhanced light and temperature, induced continuous sporing in this fern. Considering the ever-increasing interest in ferns as ornamental plants, and the growing body of evidence of toxic and allergenic effects caused by fern spores, this kind of sporing behaviour may have implications for human health.

Ferns in their natural environments usually have regular and predictable periods of spore production and release. In the temperate zones and the seasonal tropics they tend to release their spores towards the end of the growing season. In the wet tropics, where the growing season is much longer or even continuous, initiation of new fronds and maturation of older fronds take place throughout the year (Page, 1979). Most ferns have been said to show very little fluctuation in annual spore output with variations in climate, in contrast with good and bad seed years in angiosperms and conifers (Page, 1979). This does not apply to all fern species. Page (1976) pointed out that for *Pteridium aquilinum* (bracken) the spore yield can vary widely between different years. Similarly, Steeves (1959) noted that, for *Osmunda cinnamomea,* a hot dry summer is usually followed by a high degree of fertility in the following spring, whereas a cooler moister summer leads to reduced fertility. Furthermore, the onset of the reproductive phase in fern sporophytes can be demonstrated to be regulated by the interaction of several factors, such as light exposure, temperature and the nutritional status of the plant.

Field observations have suggested that the onset of the reproductive phase in ferns (as in many flowering plants) is induced by particular photoperiods, but the evidence so far published is scanty (Wardlaw and Sharma, 1963). Experiments carried out by Wardlaw and Sharma (1963) indicated that there is a more or less direct relationship between active photosynthesis and/or photoperiodic perception in the expanded leaves and the induction and development of sori in the next inner leaves of *Dryopteris austriaca.* Harvey and

Caponetti (1972), however, demonstrated that increasing light intensities inhibited sporophyll differentiation in *Osmunda cinnamomea*. Maximal initiation of sporangia occurred in total darkness in this species, so it may be that green and non-green spored ferns respond to light in different ways. There may be doubts about the importance of photoperiodic induction, but in determining the extent of the fertility in ferns, photosynthetically available radiation and the duration of exposure to light are probably of major importance. Steeves (1959) compared the incidence of fertility between *O. cinnamomea* plants in heavy wood and open areas and found a greater incidence of fertility in the latter plants. Conway (1957), Dring (1965) and Page (1976) suggested the same property in *Pteridium* (bracken), in which they found a gradual decrease in fertility with increasing degree of shade, although vegetative growth in the latter may be little impaired. The enhancement of sporogenesis by high light has recently been confirmed in experiments conducted with clones of bracken grown in high and low levels of photosynthetically available radiation (Wynn et al., 2000).

Temperature also plays a role in the onset of the reproductive phase as shown by Labouriau (1958). He found that initiation of sporangia was stimulated by exposure of the developing outermost set of *Osmunda claytoniana* fronds to a temperature of 26°C; plants kept at a lower temperature remained sterile. Similar trends were reported in *Pteridium* by Sheffield (1996) and Wynn et al. (2000).

Allsopp (1964, 1965) suggested that nutritional conditions, particularly carbohydrate supply, appear to be of greater importance for the induction of sporangia in pteridophytes than photoperiodic or similar stimuli. Several studies indicate that the nutritional status of the plant is indeed of great importance for the initiation of the spore-productive stage (Wardlaw and Sharma, 1963). Goebel (1887, 1905, 1908) and Atkinson (1896) concluded, from experiments with Onocleoid ferns, that if carbohydrate supplies are inadequate, developing leaves tend to remain in the vegetative state. Goebel (1928) found that immature sporophylls of certain ferns developed as vegetative fronds when the sterile fronds of the plant were removed. This has been repeated in numerous fern species (e.g. by Labouriau (1958), Wardlaw and Sharma (1963), and Steeves and Wetmore (1953)), who thus linked induction of sporogenous tissue to carbohydrate supply. Sussex and Steeves (1958) cultured excised leaf primordia of *Leptopteris hymenophylloides*, *Todea barbara* and *Osmunda cinnamomea*, and found that high sucrose concentrations in the medium was essential for the inception and early development of sori and sporangia in those species. They also showed that an increased supply of inorganic nitrogen promotes the onset and extent of fertility in *T. barbara*. According to Wardlaw and Sharma (1963), there is a positive relationship between the amount of photosynthesising leaf surface and the induction of fertility.

It is apparent from the above that we have some limited knowledge of what triggers sporing in ferns in natural conditions. The sporing behaviour of ferns transferred from their natural habitat to an artificial environment, e.g. a glasshouse, however, remains largely unexplored. Considering the ever-increasing

interest in ferns as household and garden ornamentals (Gress, 1996) and the fact that fern spores may cause adverse health effects in humans (Simán et al., 1999), more knowledge in this field is urgently required.

The aim of the current experiment was to compare the reproductive performance of *Polypodium vulgare* plants placed in either a seasonal outdoor environment or a constant high-temperature and high-light glasshouse environment.

Material and Methods

Polypodium vulgare plants were collected in March 1998, from stone walls along the south-east side of road B4403, running along the south-east shore of Llyn Tegid (Bala Lake) (N 52°53′, W 3°38′), Wales, U.K. *Polypodium* was chosen as it represents a widespread genus, including a broad range of horticultural favorites, such as *P. amorphum, P. cambricum* and *P. interjectum,* of similar morphology and life history (Mickel, 1994).

The plants were potted in commercial potting compost within a day of collection. Lengths of rhizome were split into two equal parts (i.e. bearing the same number of fronds in each of the two pots in a pair), and each was placed in one pot. In this way 64 pots were prepared, i.e. 32 pairs of pots containing clones. The length of the potted rhizomes varied from 1 to 5 cm, but was much the same within each pair. In order to minimise the impact on the results of the growth of any apical meristems, only median pieces of rhizome were used. All plants were allowed a six-month settling-in period (mid-March to mid-September) outdoors, after which one pot of each pair was left outdoors, to the north-east of a glasshouse in the Manchester University Experimental Grounds, Manchester, U.K. These were the "outdoor population". The other group of the plants was put inside a glasshouse (mean day temperature: 28°C, range 20–38; mean night temperature: 15°C, range 11–27; photosynthetically available radiation: c. 110 μmol m^{-2} s^{-1}), at the aforementioned Experimental Grounds. These were the "indoor population". At the start, the total number of fronds in each population was very similar. No plants were given any additional nutrients during the course of the experiment. Those outside were subject to ambient rainfall, those inside were watered regularly. During the settling-in period all fronds in 14 pots, seven in each population, died. Eight of the 14 pots belonged to matched pairs, so the aim of ensuring a genetic similarity between the two populations was still met to a high degree.

Weekly records were taken of the number of fronds in each pot with a) no sori, b) immature (green) sori, c) sporing (yellow-orange) sori and d) empty (brown) sori, from the beginning of October 1998 until June 1999 for the indoor population. The outdoor population was recorded until the end of its growing season in mid-September 1999.

The proportions of recruited fronds during the experimental period by the two populations were compared with a χ^2 test. The differences in numbers and proportions of fronds of each developmental stage in the two populations were compared numerically.

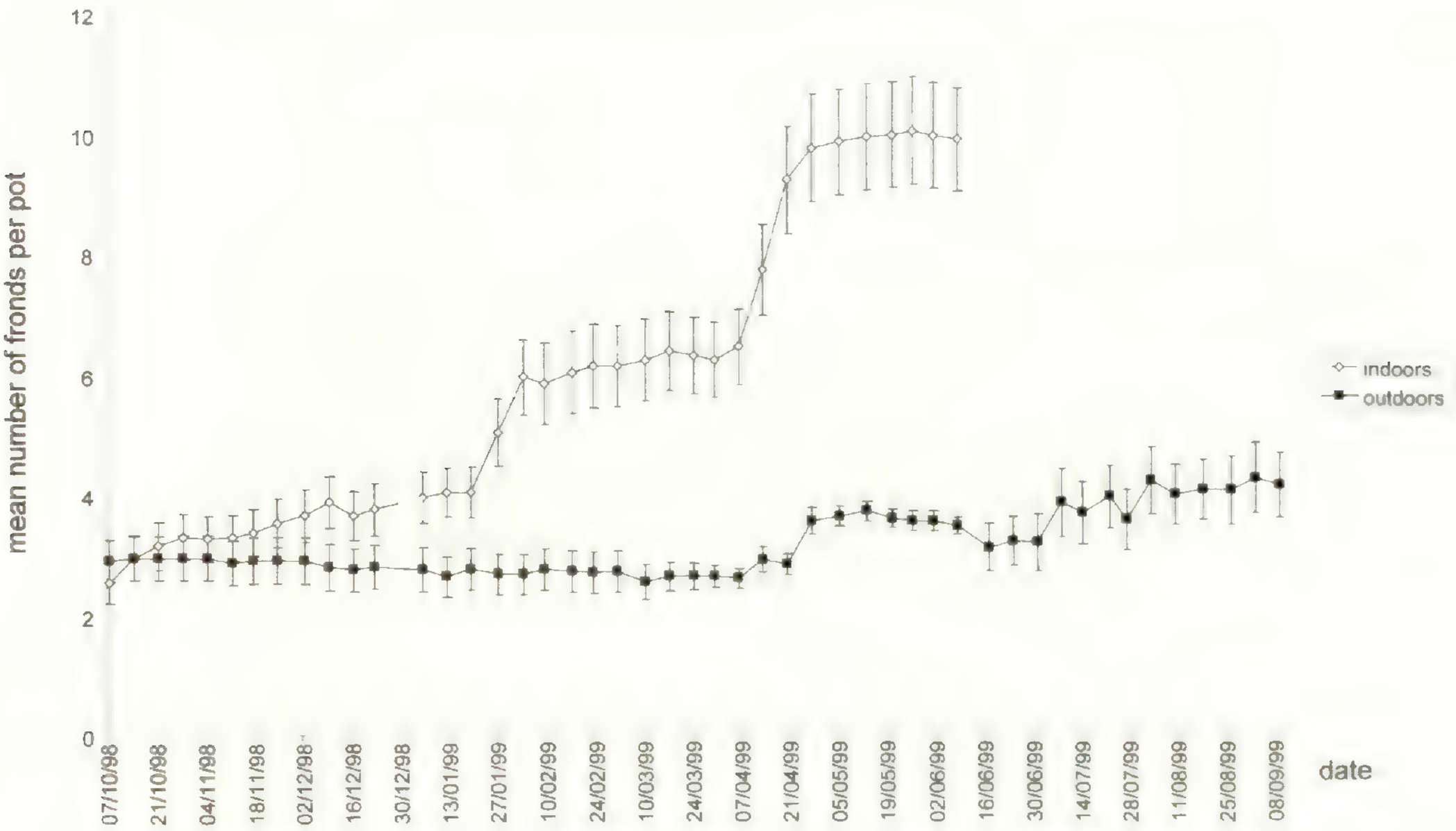

FIG. 1. Changes in the mean number of fronds per pot in two genetically matched populations of *Polypodium vulgare*. The indoor population was placed in a controlled-climate greenhouse (mean day temperature: 28°C, mean night temperature: 15°C, photosynthetically available radiation: ca 110 μmol m^{-1} s^{-1}); the outdoor population was left outside (U.K. natural weather conditions). The recruitment of new fronds in the indoor population occurred in three waves, one from October 1998 to mid-January 1999, the second from mid-January 1999 to late March 1999, and the third from early April 1999 to the end of the experiment. Error bars show standard error of the mean.

RESULTS

The recruitment of new fronds from October 1998 to early June 1999 was significantly higher in the indoor population than in the outdoor population (χ^2-test, $\chi = 127$, df = 1, $p<0.01$) (Fig. 1). During this time the indoor population increased its number of fronds more than fourfold. The recruitment of new fronds took place in three waves (Figs. 1 and 2a), each of which increased the number of fronds by a factor between 1.5 and 1.7. The outdoor population increased its number of fronds by a factor 1.2 from October 1998 to June 1999 (Fig. 1). All recruitment of new fronds in the outdoor population took place from mid-April 1999 onwards. The increase in the number of fronds in the outdoor population continued during the summer months until mid September 1999 (Fig. 3a).

The majority of the new fronds recruited in the indoor population during the course of the experiment was fertile (Fig. 2a), so the indoor population maintained a high proportion of actively sporing fronds throughout the winter. Each wave of recruitment in the indoor population began with a sudden increase in the number of initially sterile fronds; a number which decreased as sori began to appear. The proportion of fronds that remained sterile throughout the wave decreased with each wave. Thus, at the end of January 1999 the proportion of sterile fronds was 37%, in early April 1999 28% of the fronds were sterile and in early June 1999 the proportion of sterile fronds was 17%

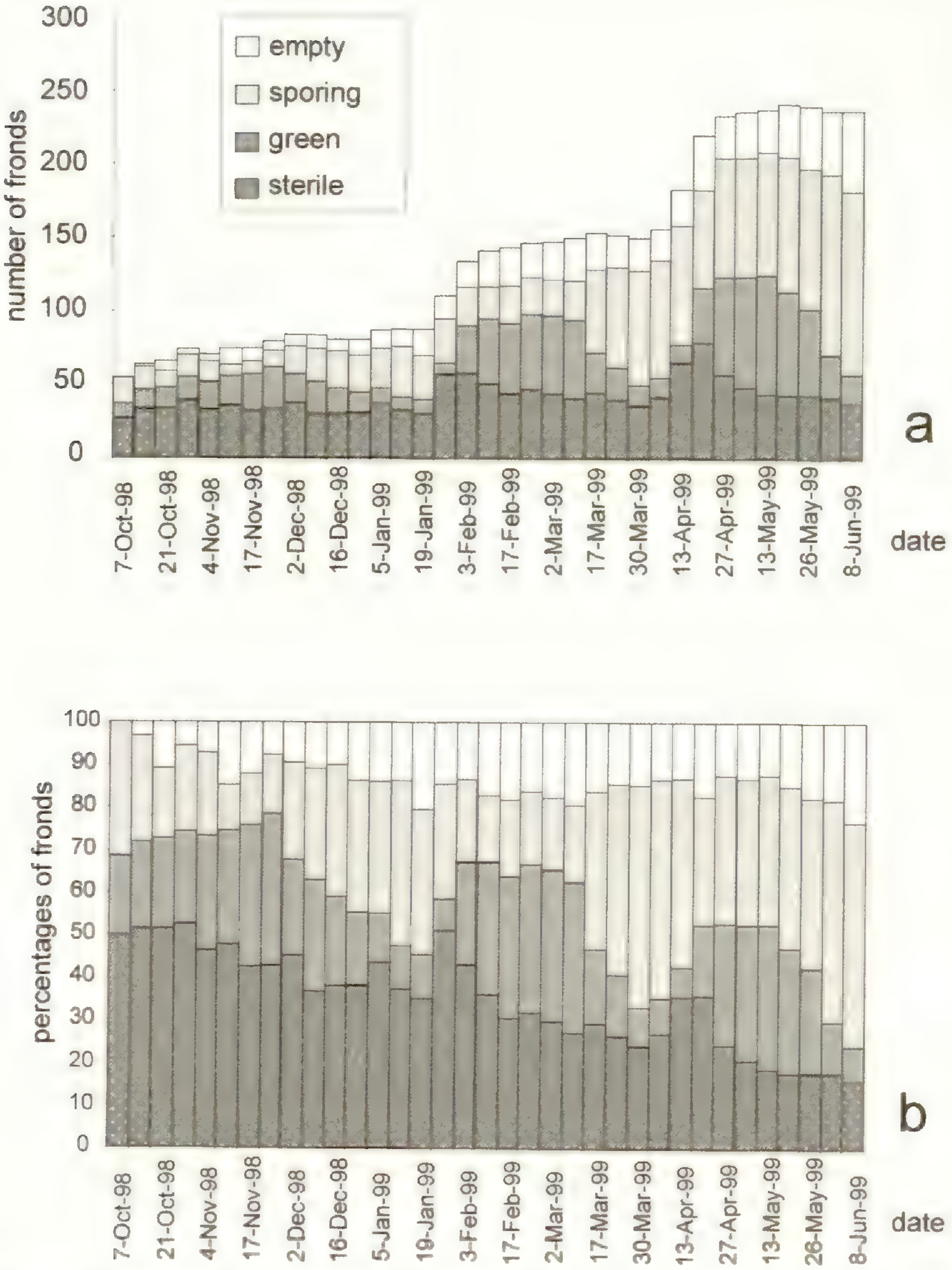

FIG. 2. Sporing behaviour in a population of *Polypodium vulgare* kept in a controlled-climate greenhouse (average day temperature: 28°C, average night temperature: 15°C, photosynthetically available radiation: ca 110 μmol m^{-2} s^{-1}) from October 1998 to June 1999. The subdivision of the bars represents i) sterile fronds and fronds with ii) green sori, iii) sporing sori and ix) empty sori, as indicated by the key in the figure. **(a)** Total number of fronds in the population (full bars) and number of fronds in each of the four groups (i.e. sterile, green, sporing, empty) for each week of the experiment. **(b)** Proportions of sterile, green, sporing and empty fronds, respectively, for each week of the experiment.

(Fig. 2b). The frond mortality represented 7.8% of the total number of fronds gained over the experimental period and was entirely due to old fronds withering and falling off.

In the outdoor population there was no net recruitment of fronds during the winter and early spring. When the new fronds started to emerge, in late April,

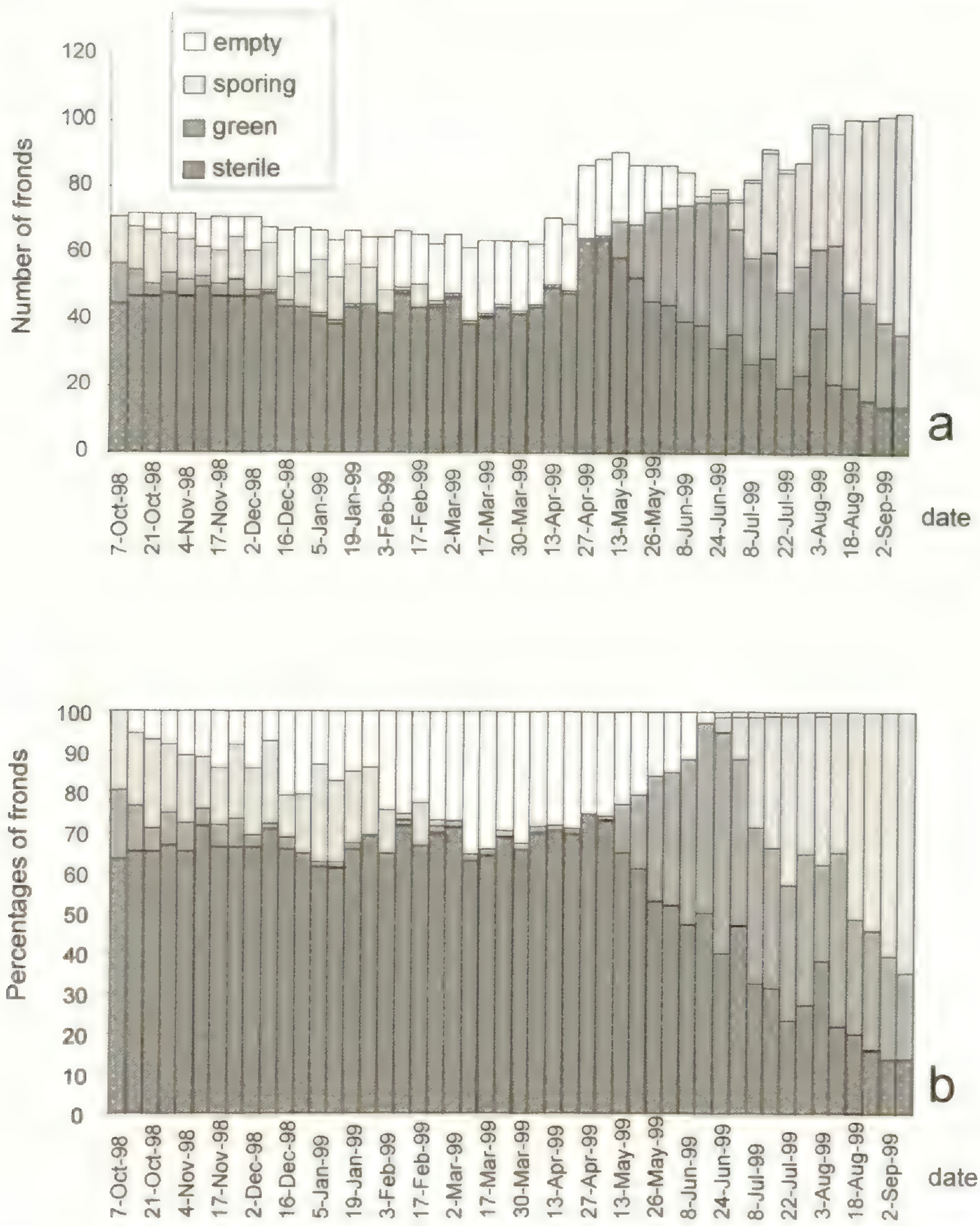

FIG. 3. Sporing behaviour in a population of *Polypodium vulgare* kept outside in the Manchester University Experimental Grounds, U.K. (natural weather conditions) from October 1998 to September 1999. The subdivision of the bars represents i) sterile fronds and fronds with ii) green sori, iii) sporing sori and iv) empty sori, as indicated by the colour code key in the figure. **(a)** Total number of fronds in the population (full bars) and number of fronds in each of the four groups (i.e. sterile, green, sporing, empty) for each week of the experiment. **(b)** Proportions of sterile, green, sporing and empty fronds, respectively, for each week of the experiment.

most of them soon turned into fertile fronds, so there was a steady increase in the number and proportion of fertile fronds over the summer (Figs. 3a and 3b). The proportion of sterile fronds decreased simultaneously so towards the end of the growing season in mid-September 1999, the proportion of sterile fronds was 17% (Fig. 3b), i.e. the same as for the indoor population at the end of its

third wave in early June 1999. The increase in number of fronds in the outdoor population was slightly impaired at two points (24/June/99 and 22/July/99) by herbivory from snails, but the fronds thus lost represented no more than 5% of the population.

Discussion

It is clear that the conditions of a warm and illuminated glasshouse stimulated the vegetative growth and spore output of the *P. vulgare* plants.

In the indoor population, the initial response to the glasshouse conditions was increased vegetative growth. At a time when the outdoor population stopped producing new fronds, the indoor population continued recruiting. Similar continuous growth has been observed in *Pteridium* grown in glasshouses (Wynn et al, 2000), but it is interesting that Thomson (2000) reports that *Pteridium* plants from four places (Honshu, Japan; Kiev, Ukraine; Bridgton, Maine, U.S.A. and Waterloo, Michigan, U.S.A.) require cold treatment (4°C) for four to eight weeks to ensure successful spring emergence of croziers when cultivated in the relative warmth of Sydney Royal Botanic Gardens (summer temp: max. 25.5°C, min. 18.2°C; winter temp: max. 16.8°C, min. 8.7°C). It seems that fluctuating temperatures warmer than those of the natural environment of a fern can have adverse effects on frond recruitment.

The majority of the new fronds emerging in the indoor population of the present study became fertile, so a high proportion of fertile fronds was maintained throughout the winter. Steeves and Wetmore (1953) concluded, after experiments with *Osmunda cinnamomea,* that the factors which determine fertility exercise their effects during the year before the leaves expanded. Assuming, in the present experiment, that each wave of recruitment created in the indoor population mimicked one growing season, we could suggest that the warm and bright indoor climate had an effect on the fertility of the second and third waves of new fronds. The proportion of fertile fronds in each of those two waves was higher than in its preceding wave. This may well be an effect of the enhanced nutritional status of the population, caused by a high production of photosynthate, which, transported as sugars to the bud primordia, might induce fertility, as suggested by Harvey and Caponetti (1972).

In its natural environment *P. vulgare* produces ripe spores from July/August. The ripening of the spores is a gradual process, occupying a period of several months. Within a single sorus some sporangia shed early and others will take longer to ripen and shed later (Wright and Wright, 1999). The persistent proportion of 10–20% sporing fronds in the outdoor population from October 1998 to March 1999 is evidence of this behaviour.

Each wave of recruitment indoors, as well as the single period of recruitment outdoors, (i.e. the growing season) increased the number of fronds by a factor of c. 1.5. This suggests that there were, at the beginning of the experiment, an equal number of dormant buds in the rhizomes of the populations and that the number of dormant buds an existing number of fronds can initiate for the next generation is restricted by something other than purely environmental

factors. This could explain the occurrence of the proportionally similar waves of recruitment.

The present study shows that by enhancing the light and temperature it is possible to interrupt the strict reproductive cycle and induce continuous sporing in a pteridophyte population. Evidence of similar behaviour has recently been obtained in another study, in which dormant *Pteridium* rhizomes produced fertile fronds within 13 weeks of being put into warm, well lit conditions (Wynn et al., 2000). Air samples taken in glasshouse and fernery environments in the UK do include ferns spores at all times of the year (e.g. Winston, 1998; Simán, 2000).

This study suggests that transfer of plants to glasshouses could benefit fern spore collectors by inducing continuous sporing in plants. There are less welcome implications of fern spore production in indoor environments, however, especially for species that do generate vastly more spores in glasshouse settings than those in natural environments. A glasshouse is an enclosed environment with little chance of biological particles being blown away by winds. This means that there is a higher risk of inhalation of fern spores in a fern-rich glasshouse than in most places outdoors. Based on the growing body of evidence of toxic and allergenic effects caused by fern spores (as reviewed by Simán et al., 1999, see also Simán et al., 2000), we suggest that some protective measures (e.g. face masks) should be taken by people who regularly work in or visit indoor fern-rich environments.

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Obituary: Warren H. Wagner, Jr. (1920–2000)

DONALD R. FARRAR

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Warren Herbert Wagner, Jr. was born on 29 August 1920 and raised in Washington D.C., the son of Warren Herbert Wagner and Harriet Claflin Wagner. His early interests in natural history took him frequently to the Smithsonian Institution, where he became acquainted with the experts, including

the eminent pteridologists William R. Maxon and Conrad V. Morton. In college at the University of Pennsylvania, he became the enthusiastic field companion of Edgar T. Wherry, author of the "The Fern Guide." Wherry was a mineralogist who became an expert on fern habitats and the first to point out the important associations of epipetric ferns with particular rock types.

Graduating from the University of Pennsylvania in 1942, Herb entered the U.S. Naval Air Corps, serving first in the Atlantic, then in the Pacific Fleet, where he was a Naval Air Navigator. In the Pacific islands he spent his off-duty hours collecting ferns and butterflies, later publishing (with David Grether) "Pteridophytes of Guam" (1948) as well as articles on the pteridophytes and butterflies of the Admiralty Islands. During this time he also flew into California, taking his specimens to E. B. Copeland at Berkeley, renowned expert on Philippine ferns. This was the beginning of an association that would bring him back to Berkeley for graduate study. While in the Navy, he also began what was to become a life-long study of the ferns of the Hawaiian Islands.

At the University of California–Berkeley, in 1945, Herb joined student colleagues Charles Heiser, Ernest Gifford, Frank Ranzoni, Vern Grant, Art Krukeberg, and others in courses instructed by G. Ledyard Stebbins, Adriance Foster, and Herb's major professor, Lincoln Constance. Copeland, although retired, was still active and advised informally on Herb's research. Also among Herb's student colleagues in systematic botany was Florence Signaigo. Herb and Florence married in 1948 and began a lifelong productive partnership in research and publication.

After receiving his Ph.D. in 1950, Herb spent a year as a Gray Herbarium Fellow at Harvard, then moved to the University of Michigan in 1951, where he remained throughout his illustrious career in teaching and research. He chaired or co-chaired the graduate programs of more than 45 Ph.D. students. He taught a variety of graduate and undergraduate courses, including his highly popular "Systematic Botany" and "Biology of Woody Plants," both of which he continued to co-teach after "retirement" (1991) through the fall of 1999. Herb's public lectures and seminars were equally popular. Few biologists have been in such demand as a visiting speaker. His *curriculum vitae* list of "invited lectures" totaled 169—since 1992!

From 1966 to 1971 Herb served as Director of the University of Michigan's Matthaei Botanical Garden. His popularity with garden clubs, amateur botanists, and conservation groups made the Gardens a center of outreach activities in this arena, as well as a center for research and display of conservatory plants. He chaired the Department of Botany in the Division of Biological Sciences from 1974 to 1977 and chaired many additional department and college committees, including the University of Michigan's Tropical Studies Committee from 1983 through 1997. He was chairman or president of nine professional societies, including the American Fern Society, American Society of Plant Taxonomists, and Botanical Society of America, and council member/trustee/advisor to dozens of organizations. He served as an editor for the Flora of North America, co-editing the Pteridophytes in volume two.

As reviewer of countless journal manuscripts, grant proposals, and botany/biology programs, he gave freely of his time while continuing to teach and maintain a research program generating over 240 research publications.

"Probably the best-known botanist ever to work at the University (of Michigan)," Herb Wagner's influence on his science was immense. He has been referred to as "the founder of modern day systematics" in reference to his seminal contributions to cladistic analysis of phylogenetic relationships. His studies in the recognition of species hybrids, their value in understanding species relationships and their role in speciation and evolution became classics. His knowledge of ferns worldwide was phenomenal and allowed unique insights into fern ecology and life history, as well as systematics. Along with awards recognizing his contributions to systematic biology (including Willi Hennig Fellow, National Academy of Science, and the Asa Gray Award of the American Society of Plant Taxonomists), his name is indelibly inscribed in cladistic literature *via* the universally recognized "Wagner Tree" representation of phylogenetic relationships.

In writing about the career of Edgar Wherry, Herb stated that Wherry "was one of those rare individuals—a real naturalist." Extended as the highest of compliments, the same could be said of Herb Wagner. His had an exceptionally keen ability to observe the small and intricate details of plants interacting with their environment. *Study the knowable. Accumulate information on the parts. With time, dedication, and an open mind, the big picture will emerge.* These gems of Herb's philosophy attributed value to all research, no matter how small the project or whom the researcher. All good data were worth getting excited about—and he did. Herb's ability to inspire others through his interest in their studies and their knowledge not only fostered independent research, but also created a legion of professionals and amateurs eager to contribute data to Herb's projects. The total productivity of this synergism, though unquantifiable, remains hugely visible.

Herb maintained a rigorous schedule of teaching, research, invited lectures and symposia presentations until just weeks before his death on January 8, 2000 at the age of 79. He was very much looking forward to participating in the 2000 Botanical Society of America symposium on the "Biology and Conservation of the Ophioglossaceae" that he helped to organize. In the summer of 1999, Herb and Florence conducted field work in Alaska and in southwestern Canada. From both places they returned with, of course, new species of *Botrychium*.

The foregoing highlights of the career and achievements of Warren "Herb" Wagner fall short in communicating the extraordinary nature of his personality, his gift for teaching, and his full influence on pteridology and pteridologists of the last half-century. The more personal reminiscences that follow portray a rare individual whose life we are all so fortunate to have shared.

ACKNOWLEDGEMENTS

Information on the early years of Herb's career were graciously provided by Florence Wagner. Factual information is taken from Herb Wagner's 1999 *curriculum vitae*. Other observations

extend from my long association with the Wagners, as a graduate student in Ann Arbor and in many subsequent field trips and discussions of plants, people, and philosophy.

Portions of this article are excerpted with permission from *Taxon* 49:585–592. August 2000, "Warren H Wagner, Jr. (1920–2000)," by Donald R. Farrar. Quotations are by Julian P. Adams and William R. Anderson of the University of Michigan in *The University Record* 55:2–4. January 17, 2000, "Warren H. ('Herb') Wagner," by William R. Anderson.

Reminiscences

Meeting Herb Wagner.—I first met Warren Herb Wagner, Jr., on the evening of 13 June 1980, at Flathead Lake Biological Field Station in far northwestern Montana. This meeting has stuck in my mind, perhaps because in many respects it typified my relationship with him. I was a new student in pteridology and had come to Flathead Lake to take his (and Florence's) four-week-long fern course. Earlier that year I had phoned Herb to tell him that I had enrolled in his course and to let him know about a new, unnamed fern hybrid I had found in the Shawnee Hills of southern Illinois, a cross between the walking fern (*Asplenium rhizophyllum*) and the maidenhair spleenwort (*A. trichomanes*). As it turned out, Herb was a few days late for the course because of a conflict with the final days of the International Botanical Congress in Vancouver. He finally arrived at the biological station about 10:30 p.m., when I was alone in the lab and identifying plants. The lab door suddenly opened and Herb burst into the room. He walked about half its length before acknowledging my presence. We introduced ourselves. He explained that he had been collecting moonworts all day en route to the field station, but it didn't seem like it to me. Instead of being exhausted after a long day of fieldwork, he was animated and lecturing me about the biology of moonworts and ferns in general. I listened spellbound and thrilled that he would take the time to explain it all to me. "Let's see your hybrid fern," he demanded. "It's in my cabin," I replied, "and my cabin is a long way away, and it's pitch-black outside, and I've lost my flashlight. Can I show it to you tomorrow?" Then—and I'll never forget this—he gave me the most odd supplicating look, and with hands clasped together as if praying, he said, "Won't you *please* go get it—*now*!" How could I refuse? I stumbled back to my cabin in darkness, found the specimens, and retraced my steps to the lab. Herb examined the specimens. "Yes, that's it! *Asplenium rhizophyllum* × *A. trichomanes*. Congratulations!" He shook my hand; I had received his imprimatur. Then, without further ado, he described how he wanted the lab rearranged—by me, that is. Tables, benches, plant presses, cardboard dividers, microscopes—all these were to be moved according to a plan that he had devised while I was retrieving the specimens. After explaining where everything should be relocated, he said, "Ok, gotta blow!" and he left the lab as abruptly as he had entered. I was once again alone in the lab, excited by what I had just learned about moonworts and spleenworts, and too motivated to mind having been pressed into service.—*Robbin C. Moran, The New York Botanical Garden, Bronx, NY 10458-5126.*

Herb at "Bug Camp".—My first memory of Herb Wagner is from Spring, 1952. During my senior year at the University of Michigan, about to graduate with a major in Biological Science, I worked as an undergraduate lab assistant in botany. That job involved lugging five-gallon bottles of distilled water, spraying the greenhouse for insects, and other tasks. One day I was called in to meet a new faculty member, Dr. Warren H. Wagner, Jr. He asked me to be his graduate assistant that summer at the bug camp, the University of Michigan Biological Station at Douglas Lake. With no other plans, I agreed.

That marked my beginning as a botanist. I assisted Wagner in courses in Phycology and Pteridology. To be a graduate student, I had to take a course, so I signed up for an independent study with him. Since the genus *Equisetum* was well represented around the bug camp, Wagner assigned me to work with its taxonomy. Herb showed himself to be an enthusiastic, knowledgeable field botanist. I well remember, after a hard day bent in a half-crouch searching out *Botrychium*, stopping with him for ice cream on the way back to Douglas Lake. And, I remember evenings all of us gathered around a piano to hear him play. Most evenings were spent in the lab until 11 p.m. or so, working with Herb, on the materials gathered during the long days in the field.

That summer, Herb advised me to go to some other university for my master's degree, to widen my exposure to the whole field of botany, with the understanding that I would return to Michigan to work for my doctoral degree under him. I went off to Florida State University as a graduate research assistant on a tidal marsh study, then to the University of California, to study the anatomy of *Equisetum* stomata with Dr. Adriance Foster and get an M. A. in Botany. After a stint in the U. S. Army, I returned to Ann Arbor, to my mentor, Dr. Warren H. Wagner, Jr., and to the taxonomy of *Equisetum.—Richard L. Hauke, 900 N. Stafford St., Apt. 1103, Arlington, VA 22203-1844.*

Unflappable Herb.—If you know something of Herb's activities during World War II, it is no surprise that he was unflappable. He had been an officer in the U. S. Navy, a navigator on air flights, and for a time was based on Guam. Like the pilots, he was not called upon to fly every day; on his days off he collected butterflies and ferns. After the end of the war, he and David F. Grether published an account of the pteridophytes of Guam based principally on their collections and those of other U. S. military personnel. (Occ. Pap. Bishop Mus. 19(2):25–99. 1948). He was a native of Washington, DC, and his butterfly collection was given to the Smithsonian Institution.

The best collecting for butterflies, Herb found, was beyond the American defense lines that were maintained by Marine guards and patrols. Some well armed Japanese troops were still at large beyond those lines, along with numbers of their fallen colleagues who were very attractive to butterflies. Because of the danger, the Marine guards didn't like Herb's excursions, but as an officer, he was able to go where he wanted. A butterfly net was of the utmost importance to those excursions. It was the equivalent of a white

flag that signaled the bearer's non-combatant status. Even so, there must have been a considerable element of danger, although Herb said he was never shot at by the Japanese while carrying a net. (A plant press, even loaded with ferns, did not confer the same protection, presumably because its function was unknown to the Japanese soldiers.)

In Ann Arbor, Herb's spring wildflower course was always popular. His humor, knowledge, and enthusiasm were delightful. Who could forget his earnest description of a hand lens and how it was to be used and worn around the neck? He then pulled from within the lectern a large hand lens attached to a red ribbon so wide that it was practically a sash! Although Herb was generally unflappable, I do remember one exception that was, depending upon your point of view, hilarious.

The year I assisted him, his lectures were presented in three classrooms that had been converted into a long, narrow lecture hall. The slide projector, which I manned, was placed well back in the hall, perhaps 40 feet from the desk, lectern and screen at the front. In order to signal for a change of slides, Herb would hold a wooden pointer vertically and drop it dramatically on the concrete floor next to his feet. Although none of us knew it at the time, the repeated thunk was not popular with the Dept. of Geology professors who had offices on the floor below.

One afternoon, Herb called for darkness. The shades were drawn while Herb turned out the room lights. I turned on the projector and put the first slide on the screen. Naturally, I was looking at the projector when Herb dropped the pointer to request the second slide. There was a tremendous detonation in the front of that quiet room. My head snapped up in time to see Herb still coming down behind the desk after what must have been a terrific leap, nearly a pole vault. He strode over to the door, clicked on the lights, took out and lit with trembling hands one of the small cigars he smoked at that time, inhaled deeply, and said "Take a five-minute break." Of course, the students could not contain themselves. By evening, most of the graduate students knew what had happened: one of them had taped a few caps from a cap pistol to the blunt end of the pointer. After the recess and cigar, Herb's lecture and slides continued as before, punctuated only by an ordinary thunk. The Geology professors were not at all amused.—*David B. Lellinger, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560-0166.*

Graciousness and Support.—Like most graduate students back in the 1970's, it wasn't long before beginning my study of ferns that I was introduced to Herb Wagner. My first impressions were that he was brilliant, dynamic, mesmerizing, and exactly the type of scientist that I wanted to be. Of course, that wasn't possible because he was certainly one of a kind. He reviewed one of the first papers that I wrote concerning meiotic studies in *Ceratopteris*. I remember that the review was less than favorable about some aspects of the study and of the way that it was presented. He was right, of course, but for some reason, perhaps extensive re-writing, it eventually was published.

A couple of years later, I wrote a paper that made an attempt to propose an alternative explanation for the pattern of reticulate evolution that Herb had described for Appalachian *Dryopteris*. This was the last piece of my doctoral dissertation. I soon graduated and while I was at work as a young assistant professor, I actually received a reprint request for this paper from Hugh Iltis with a big "congratulations" scrawled on it! But any glory was to be short-lived. A couple of years later, during a meeting of the Southeastern fern group at the Duke Marine lab, I remember sitting quietly in the front row as Herb methodically destroyed my brash interpretation of Appalachian *Dryopteris*. I was humbled, but not humiliated. He was far too gracious for that.

Although I was not one of his students in any type of academic lineage, he was a constant and helpful influence in my career. Letters of recommendation for jobs and promotions, reviews of my academic department, and occasional suggestions spanned the course of over 20 years. My last personal encounter with him was during a visit to Knoxville in the late 1990's where he gave an inspiring seminar to a group of faculty and students. I was teaching a sophomore genetics class that semester and made a point of inviting my students to hear his seminar. Although they weren't botany majors and generally had very little interest in plants, it was obvious that he had not lost his charm, dynamism, and ability to mesmerize even these supposedly disinterested young people in his story about moonworts! I keep a small picture of Herb behind me in my office and often turn and sneak a peek at him for inspiration when I am trying to figure out how to do a better job of preparing a lesson or writing up a laboratory exercise.—*Les Hickok, Department of Botany, The University of Tennessee, Knoxville, TN 37996.*

Humanity and Professionalism.—He combined intellect with a humanity and friendliness. Herb Wagner, as we called him when he befriended us, traveled widely and infected nearly everyone he met with an enthusiasm for life and for inquiry into the natural world.

His influence upon me came early in my professional career. While a student at the University of Kentucky between 1961 and 1963, Herb had already visited the campus, and as always, took a field trip into the Kentucky hills. I was told that an old man walking along a road of a cove yelled, "Hey, what are you fellers doing down there?" Herb's immediate response, "We're just botriculating." And though suspicious of revenuers, the old man responded, "Oh well, go right ahead."

It was shortly after I arrived in Cullowhee in 1969 that Herb made a visit to Highlands Biological Station in preparation for the fern conference in 1970. He had come across a paper that Jim Horton and I published listing new county records that included *Asplenium heteroresiliens*, a hybrid on marl known only from the coast. He visited our herbarium and in his instructive but gentle way said, "I see why you identified *Asplenium trichomanes* for *heteroresiliens*, but it isn't." Our somewhat depauperate and poorly dried specimen didn't exactly match either species. Herb also got me

to be more careful in my identification of other species, pointing out that what appeared to be *Populus grandidentata* growing beside the science building was something else, which we later identified as *Populus* × *canescens*.

In the early 1970's Herb was President of the American Fern Society, popularizing the profession. About this time I had a free evening and took in the movie, "A New Leaf," a spoof of a millionaire and his attraction to a wallflower type of lady who happened to be a student of ferns. To my amazement, she came to the point of verifying her identity that the tropical fern she discovered in Alaska was confirmed by "Dr. Wagner at the University of Michigan." Such was his fame as it expanded beyond the ivory tower into society in general.

Herb visited Cullowhee many times. On his last trip here in April, 1997, not only did he give the visiting scholar lectures but also wanted to return to some nice place before he returned home. His 4 p.m. seminar that Friday afternoon was so well received that a couple of visitors, Bob Dellinger, local naturalist, and Gary Kauffman, USDA Forest Service botanist, asked to tag along with us the next day to the Joyce Kilmer area. Although Herb had indulged in country ham the previous days (he was supposed to be on a low salt diet) and was a bit tired from the activities the previous two days, he delighted in finding *Carex austro-caroliniana* with peduncles several centimeters long. Such was his exuberance over those things he found fascinating.—*Dan Pittillo, Department of Biology, Western Carolina University, Cullowhee, NC 28723.*

In His Element.—When I think of Herb, I tend to think of him in a field trip setting. For instance, when he was invited to the annual meeting of the Field Botanists of Ontario in Midland, Ontario a number of years ago, I remember the lead car leaving the sandy gravelly country road and entering an open sandy field with a few copses of junipers. In what resembled a "cops and robbers" film on T.V., the lead car had not come to a full stop when Herb rolled out the front door of the car. He took a giant step towards the copse and was almost immediately flat on the ground under the prickly Juniper. Suddenly there was a victory call for all and sundry within 200 yards—"Botrychiums." All Herb's followers were on the spot in a few seconds and each looked in marvel at some very small specimens between the juniper and the sand—a gap of less than a foot. Herb was in his element in the field, and his disciples enjoyed every minute of it!—*D. M. Britton, Professor Emeritus, Department of Molecular Science & Genetics, University of Guelph, Guelph, Ontario, N1G 2W1 Canada.*

One of the All-time Giants.—My first contact with Herb Wagner came about in early 1978 when I was at the University of Cambridge, England. I had been intrigued for some time by a note in Herb's paper on spores in relation to fern phylogeny (Ann. Missouri Bot. Gard. 61: 346. 1974) on the presence of large spores in ferns in the higher altitude rainforests of Hawaii not being linked to polyploidy. I had noted that in the New Zealand species of *Grammitis* (Grammitidaceae) there is a trend towards the production of

larger spores, and frequently fewer spores per plant, in the species occurring at higher altitudes and latitudes. Although few chromosome counts are available this does not seem to be linked to polyploidy. I had assumed that this wasn't a parallel of the strategy of higher plant taxa that at higher altitudes produce fewer and larger seeds to contain resources needed during longer periods of dormancy, because dormancy is not an issue with Grammitid spores, which are photosynthetic. Perhaps Herb's "selection for precinctiveness" was working here? So I wrote to him concerning my observations; he promptly replied that he thought it was, and that in Hawaii, for example, any spore or any propagule that is especially likely to be carried by wind is going to lose out. His observations on butterflies had some part in his logic, with the most conspicuous Hawaiian butterfly being a very sluggish inhabitant in mountain valleys, rather than ridges, where it was likely to be blown away. I'm still very much undecided about selection for precintiveness in Grammitidaceae after further work, as the high altitude New Guinea species do not show the same increased spore size as the New Zealand ones, but Herb's prompt, encouraging and discursive response to a junior unknown pteridological correspondent made a deep and lasting impression on me that here was one of the all-time giants of pteridology.

Nine years later, when I had moved to the Royal Botanic Gardens, Kew, I finally met both Herb and Florence at the International Botanical Congress in Berlin in 1987, and the impression I had of Herb from his correspondence was strongly reinforced. He enjoyed ferns and people and talk, and I really envied all of his students for having such a stimulating mentor! A talk with Florence at Berlin turned up a surprising coincidence—one of my M. Sc. supervisors at the University of Auckland, New Zealand, Prof. Jack Rattenbury, had been best man at Florence and Herb's wedding—it's a very small botanical world.—*Barbara Parris, Fern Research Foundation, 21 James Kemp Place, Kerikeri, Bay of Islands, New Zealand.*

Tutor and Friend.—Herb Wagner was my tutor and friend during the last dozen years of his life and he assisted me in gaining the skills needed to study the Hawaiian ferns. Herb worked with the Hawaiian fern flora for more than half a century, during which time he greatly increased the knowledge of its diversity and biology. He described more than three dozen new species, varieties, and hybrids. With his contributions, our organized understanding of the Hawaiian ferns was much advanced.

Herb made several long visits to Hawaii during which he went on extensive field trips and spent much time in local herbaria. During these visits he taught courses reviewing the Hawaiian ferns and took his students on field excursions. A remarkable and enthusiastic teacher, the passionate sharing of his deep knowledge of Hawaiian ferns inspired many in Hawaii to study this neglected group of plants. His lectures and seminars were remarkable for his enthusiastic, thorough, and effective presentation of material to students with only a minimal knowledge of pteridophytes. I will remember the ballet like pirouettes he would do at the blackboard when emphasizing a point,

and I remember the vast collection of anecdotes and jokes he used to enliven a lecture and to help students remember the subject under discussion. He would not let the decreasing physical vigor he experienced in later years interfere with active field work. On his return to Ann Arbor from Hawaii he always left behind a whirlwind of enthusiasm for the study of Hawaiian ferns.

Florence Wagner's collaboration and organizational abilities allowed the Wagner family to effectively pursue the twin goals of teaching and research. Many of us in Hawaii will fondly remember a day's end with Herb and Florence, where a good dinner, cocktails, stimulating conversation, and enjoyable company combined to make for a very pleasant evening.

He is missed by all who knew him in Hawaii. His stimulating visits will be missed and the time he spent here will be remembered here with nostalgia.—*Daniel D. Palmer, 1975 Judd Hillside, Honolulu, HI 96822.*

Missing Herb.—I miss Herb too much to find words to describe how miserable I am at knowing he is not at the end of the phone needing a this or a that, or welcoming my weird plant ID, or to confirm one of those peripheral vegetative idioblastic forms that my heart hoped might be something new. I remember the summers he visited Iowa Lakeside Lab, the AFS field trips, particularly the one in northern Michigan. I did not know Herb from Michigan as many did, as he was my academic grandfather—and father in Iowa by remote-control, a fitting way to be for us hybrid studying types. He thought that my following Darwinian fates of individuals and natural history approach to fern reproduction and population structure was significant when most around me spoke only of things homoeologous, bar codes of molecules on gels, bootstrapping clades, and such. In comfort, Herb offered that he often no longer knew what cladistic people meant with the language they used to describe his ground-plan divergence method he created so long ago. I only wanted to be a teacher, and Herb's way with a gathering of students still is my exemplar. We will all miss him; he will always be with us.—*James H. Peck, Department of Biology, University of Arkansas—Little Rock, Little Rock, AR 72204.*

A Personal Gift.—I didn't go to the University of Michigan to study with Herb Wagner. My interests were in plant physiology, but in my first semester a fellow graduate student, Bob Faden, who was then a student of Herb's, secured an invitation for me to accompany the annual Wagner entourage to the hills and canyons of southern Ohio and Kentucky. Herb had just published papers on the independent occurrence of gametophytes of *Vittaria* and *Trichomanes* in the eastern United States. We had no trouble finding great mats of *Vittaria* gametophytes and, with some squirming on our backs under overhanging sandstone cliffs, we also found the green, threadlike gametophytic filaments of *Trichomanes*. Returning with collections of these intriguing plants, I was thrilled and convinced that with my growing physiological expertise I would coax them in culture into producing sporophytes and would resolve the mystery of their independent (without sporophyte production) occurrence in the wild. Nearly 40 years later I still work on that.

After two years of graduate school I was having doubts about my future in academia. With considerable trepidation I informed Herb of my intention to take some time off and join the Peace Corps. To my surprise, he supported my decision, but then gently reminded me that I was now the "world's expert" on independent gametophytes and I "owed it to science" to stay one more year to publish what I had learned. By the time I did that, of course, my thinking had changed and I was forever hooked on fern gametophyte biology. Herb probably expected that outcome, but it wouldn't have happened without his encouragement and confidence in me.

The excitement of field trips with Herb and Florence remain highlights of my graduate years at Michigan, as do warm memories of holidays at the Wagner home, cutting out snowflakes or whatever project Florence had designed for their "extended family" of graduate students. This nurturing of an Ozark farm boy a long way from home made a difference. It was a personal gift. Yet I know it was only one of many such personal gifts, bestowed on many others as well, by Herb and Florence. For those gifts we all say thanks!—*Don Farrar, Department of Botany, Iowa State University, Ames,* Iowa 50011.

American Fern Journal 92(2):50–64 (2002)

Bibliography of Warren Herbert Wagner, Jr.

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ABSTRACT.—This bibliography contains 395 entries, all the scientific publications of Warren H. Wagner, Jr. The bibliography is based on a list maintained by Prof. Wagner, with additional comments and entries by Dr. Florence S. Wagner and Dr. Richard Rabeler. It includes abstracts, ephemera, reports, and reviews. The bibliography is in rough chronological order of publication. Exact publication dates were found for most journal articles. However, month or estimated dates were used for other publications, especially books, which usually are not datable within a year. All the entries are contained in a searchable database now maintained by Florence S. Wagner. The database categorizes the publications according to their subject matter and includes the more or less exact publication dates that are the basis for the chronological list.

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The Mating Systems of Some Epiphytic Polypodiaceae

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ABSTRACT.—Genetic loads, estimated from sporophyte production by isolated gametophyte cultures, indicate mating systems of intragametophytic selfing in *Campyloneurum angustifolium* (in part), *C. phyllitidis*, *Phlebodium aureum* and *Phymatosorus scolopendria*, and intergametophytic mating in *C. angustifolium* (in part), *Microgramma heterophylla* and *Polypodium pellucidum*. Polyploidy characterizes the intragametophytic-selfing species, whereas the intergametophytic-mating taxa are diploid. The duplicated loci of polyploid taxa may mitigate the expression of recessive lethal alleles caused by intragametophytic selfing, whereas genetic load probably maintains the mating systems of the intergametophytically mating taxa. Enzyme electrophoretic patterns of fixed heterozygosity support allopolyploid origins of *C. phyllitidis* and *P. aureum* and confirm their intragametophytic mating systems. Antheridiogens, present in both groups, may promote intergametophytic mating in diploids through promotion of the early development of male plants in gametophyte populations and bisexuality in isolated gametophytes of polyploids if these gametophytes delay or do not attain insensitivity to their own antheridiogen. In the polyploids, antheridiogens may also alleviate low genetic variability through promotion of occasional outcrossing. The perennial, clone-forming habit of epiphytic Polypodiaceae increases the duration and the physical space occupied by derivatives of a single spore, thus expanding the chance of interaction with a later migrant. Genetic load, duplicated genes, and antheridiogens, together with a perennial and clone-forming gametophyte growth habit, interact to produce successful breeding strategies of these epiphytic species.

Three mating systems have been documented in ferns: intragametophytic selfing, intergametophytic selfing, and intergametophytic crossing (Klekowski, 1979). The more general term intergametophytic mating refers to either or both of the last two systems when it is not possible to determine whether cross-mated gametophytes are from the same (intergametophytic selfing) or different (intergametophytic crossing) sporophytes.

Because gametophytes are potentially bisexual, it had been thought that intragametophytic selfing was predominant in homosporous ferns (Klekowski, 1979), and high selfing rates have been reported in some diploid homosporous ferns, especially those with subterranean gametophytes (e.g., Soltis and Soltis, 1986a) and in some pioneering species (Crist and Farrar, 1983). Intragametophytic selfing also seems to be the trend in species that are polyploid with respect to other species in a genus. Possibly the duplicated loci

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of polyploid species mitigate the problem of recessive deleterious allele expression associated with selfing (Klekowski and Baker, 1966; Masuyama and Watano, 1990). Intragametophytic selfing may also reflect a genetic bottleneck associated with the origin of polyploid species events. However, isozyme evidence has shown that intergametophytic mating is the most common breeding system in diploid homosporous ferns with above-ground gametophytes (Haufler & Soltis, 1984; Soltis & Soltis, 1986b; 1990).

Reproductive behaviors are affected by several factors. Inbreeding depression resulting from recessive lethal genes (expressed as genetic loads in this study) seems to be the primary factor preventing intragametophytic selfing in many species (Haufler et al., 1990). Failure to attain bisexuality and asynchronous maturation of antheridia and archegonia also promote outcrossing in potentially bisexual gametophytes (Klekowski, 1968). Functional unisexuality is augmented by antheridiogen systems that stimulate precocious antheridium formation in some gametophytes in a population while allowing others to remain unisexually female (Näf, 1979). Antheridiogen can also induce previously buried spores to germinate to form gametophytes that reach the surface and produce sperm capable of fertilizing above-ground plants (Voeller, 1971). Variation in growth habit including indeterminate growth, branching, gemma production, and production of perennial gametophyte clones can also influence reproductive behavior by increasing the probability of interaction between distant gametophytes and between plants established at different times (Chiou & Farrar, 1997b; Chiou et al., 1998; Dassler & Farrar, 2001).

Comprehensive research on fern reproduction is limited, especially in epiphytic ferns (Werth & Cousens, 1990) whose gametophytes exist in very different environments from those of terrestrial species. The habitat of gametophytes of epiphytic species is often within dense bryophyte mats where interaction between gametophytes *via* antheridiogen or sperm transfer may be significantly hindered compared to gametophytes of terrestrial ferns on less complex surfaces (Dassler, 1995; Dassler & Farrar, 2001). On the other hand, bryophyte mats may maintain free water, the medium for sperm, available for longer periods and thus promote fertilization over longer distances than substrates lacking bryophytes.

Most species of Polypodiaceae are epiphytic. The presence of antheridiogen systems (Chiou & Farrar, 1997a) and clone-forming, perennial growth habits of gametophytes of several genera of this family (Chiou & Farrar, 1997b) have been documented. Their mating systems, however, have not yet been fully described. This report further investigates the mating systems in selected Polypodiaceae species using evidence from genetic load and enzyme electrophoresis. Results of growth habits and antheridiogen systems are interpreted with regard to the reproductive biology of these species.

Materials and Methods

The materials used in this study follow. Greenhouse materials and those collected by Chiou and Farrar were used for genetic load studies. Materials

for isozyme studies came from the four Florida localities indicated below by two-letter abbreviations. Vouchers are held at Iowa State University.

Campyloneurum angustifolium (Swartz) Fée—ISU greenhouse (origin unknown) (*Farrar 02-1-8-1*) and Marie Selby Botanical Garden, Sarasota, Florida (origin unknown; *Chiou 14337*).

Campyloneurum phyllitidis (L.) C. Presl—ISU greenhouse (2 collections, origin unknown) (*Farrar 02-1-8-2*), Castellow Hammock (CH), Fakahatchee Strand State Preserve (FS), and Jonathan Dickson State Park (JD).

Microgramma heterophylla (L.) Wherry—ISU greenhouse (Florida) (*Farrar 02-1-8-3*).

Phlebodium aureum (L.) J. Smith—Fakahatchee Strand State Preserve (FS) (*Chiou 14342*), Jonathan Dickinson State Park (JD) (*Chiou 14300*), Tosohatchee State Reserve (TS).

Phymatosorus scolopendria (Burm.) Pic.-Serm.—Hawaii (*Farrar 92-8-20-1*).

Polypodium pellucidum Kaulf.—Hawaii (*Farrar 92-8-19-3*).

Spores were first sown and gametophytes grown in multi-gametophyte cultures on agar-solidified mineral media in plastic petri dishes (Chiou & Farrar, 1997a). Cultures were maintained under continuous, white fluorescent illumination of 2000–3000 lux and at a temperature range of 20–24°C.

Genetic load (presence of sporophyte-lethal alleles) was estimated by comparing isolated-spore and isolated-gametophyte cultures with paired-spore and paired-gametophyte cultures. These cultures were grown in compartmentalized plastic sheets ("jelly-molds"), each with 20 cells containing about 6 ml of agar medium. In each sheet, a single spore was transferred directly into each of 5 cells (isolated-spore cultures), and a one-month-old gametophyte which was still asexual was transferred from multigametophyte cultures into each of another 5 cells (isolated-gametophyte cultures). The other 10 cells of each sheet received two spores (paired-spore cultures) and two gametophytes (paired-gametophyte cultures). Five such culture sheets were separated by clear, flat, plastic sheets and stacked in transparent plastic boxes (vegetable crispers) for a total of 50 replicates of each culture type for each species. Gametophytes grown from spores isolated before germination served to test the possibility that gametophytes transferred from multigametophyte cultures to isolate and pair cultures might have experienced gametophyte interactions before transfer. The light intensity of these cultures was maintained between 1500 lux (bottom layer) and 3500 lux (top layer). Plants were watered with distilled water every two weeks after the gametophytes were 4 months old.

Whether sporophytes were produced by syngamy was determined by examination with a compound microscope. Genetic load was estimated by counting the percentage of bisexual gametophytes failing to produce

sporophytes (Peck et al., 1990). The five layers of sheets in each box were designated as blocks, and spore-isolated cultures vs. gametophyte-isolated cultures were designated as split plots. For *C. angustifolium*, *C. phyllitidis*, and *P. aureum*, since two sources of spores were used, a Latin Square was designed. Five treatments were "A" and "B" (isolated plants of two different sources), "AA" and "BB" (paired plants from the same source), and "AB" (paired plants from different sources). Isolated gametophytes were removed when sporophytes were formed or 8 months after spore sowing and examined microscopically to determine their sexuality. Development of gametophyte sexuality in multi-plant cultures was also monitored (Chiou & Farrar, 1997a).

For isozyme study, ten sporophytes each of *C. phyllitidis* and *P. aureum* were collected from each location. These sporophytes were at least 50 m apart for *P. aureum*, and 10 m apart for *C. phyllitidis* which was less widerspread. The grinding method and buffer of electrophoresis followed Farrar (1990). Starch-gel electrophoresis and staining were conducted following Ranker et al. (1989). Thirteen enzyme systems were scored, including aconitate hydratase (ACO), adolase (ALD), fructose-biphosphatase (FBP), glutamate oxaloacetate transaminase (GOT), hexokinase (HK), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), shikimate dehydrogenase (SKDH), and triosephosphate isomerase (TPI). To score the isozyme patterns of tetraploid species, Werth's (1989) method was followed, in which the most anodal region of activity of each locus pair is given a numeric abbreviation of "1" (e.g., Hk-1) and members of a locus pair are abbreviated with letters, with "a" designated as the more anodal region of activity (e.g., Hk-1a vs. Hk-1b). Isozyme data were analyzed with BIOSYS-1 (Release 1.7, Swofford and Selander, 1989).

Results

Sex Expression.—In multi-plant cultures female gametophytes were present at 45 days and outnumbered male gametophytes in all species tested (Chiou & Farrar, 1997a). A predominance of female plants continued through 75 days for all species, and through 90 days for all species except *Phlebodium aureum* and *Polypodium pellucidum*, when bisexual plants became dominant. A relatively small number of male gametophytes (<10%) were present in *M. heterophylla*, *P. scolopendria, and P. pellucidum* by 45 days, in *C. phyllitidis* by 60 days, and *in C. angustifolium* and *P. aureum* by 75 days. The percentage of gametophytes that were male remained low (maximum = 23% in *P. pellucidum* at 60 days) throughout the experiment for all species.

In single-plant cultures, the percentages of males, females, and hermaphrodites were not significantly different between the two spore sources of *C. angustifolium*, *C. phyllitidis*, and *P. aureum* (data not shown). Differences between the isolated-spore and isolated-gametophyte cultures at 8 months

TABLE 1. Sex expression (%) of Polypodiaceae species in isolated spore and isolated gametophyte cultures at 8 months after sowing spores.

Treatment	Male	Female[1]	Bisexual[1]
Campyloneurum angustifolium			
Spore	19	0	81
Gametophyte	6	0	94
Campyloneurum phyllitidis			
Spore	17	41(a)	42(b)
Gametophyte	8	11(b)	81(a)
Microgramma heterophylla			
Spore	14	0	86
Gametophyte	6	0	94
Phlebodium aureum			
Spore	0	3	97
Gametophyte	0	3	97
Phymatosorus scolopendria			
Spore	0	0	100
Gametophyte	0	0	100
Polypodium pellucidum			
Spore	7	85(a)	8(b)
Gametophyte	4	35(b)	61(a)

[1] Different letters in parentheses for each gender of each species indicate significant differences in Duncan's multiple test (95% confidence limits).

after sowing spores were present only in *C. phyllitidis* and in *P. pellucidium* (Table 1). Whereas few or no female gametophytes remained at 8 months in other species, in *C. phyllitidis* and in *P. pellucidium* female gametophytes were still common, especially in cultures from isolated spores.

The sexual expression of gametophytes was not significantly different (95% confidence limits in Duncan's multiple test) among different layers within cultures boxes except for *C. angustifolium*, where significantly more male gametophytes developed in the lowest (and darkest) layer.

GENETIC LOAD.—At 8 months after sowing spores, sexual sporophyte production was lower in the isolated cultures (of both isolated-spore and isolated-gametophyte cultures) than in paired cultures of *M. heterophylla* and *P. pellucidum* (Table 2). There was no significant difference between isolated and paired cultures of *P. aureum* and *P. scolopendria*.

In *C. angustifolium*, gametophytes from source B failed to produce sporophytes in both isolated-spore and isolated-gametophyte cultures at 8 months after sowing spores, whereas 83% of isolated plants from source A produced sporophytes. Thus the inferred genetic load of source B was extremely high, whereas the genetic load of source A was quite low.

In *C. phyllitidis*, no difference was observed in sporophyte production between gametophytes from the two spore sources. There also was no significant difference between sporophyte production by single gametophytes isolated as young plants from multi-gametophyte cultures and any of the

TABLE 2. The percentage of gametophytes producing sporophytes sexually and the estimated genetic load expressed by bisexual gametophytes of 8 month old spore-isolated and gametophyte-isolated cultures of Polypodiaceae species.

	% Producing sporophytes			Est. genetic load[2]		
Source[1]	Spore[3]	Gametophyte[3]	Average[3]	Spore[3]	Gametophyte[3]	Average[3]
Campyloneurum angustifolium						
A	77(a)	90(a)	83(ab)	16(b)	8(b)	12(b)
AA	92(a)	96(a)	94(a)	–	–	–
AB	73(ab)	86(a)	79(b)	–	–	–
BB	44(c)	48(bc)	46(c)	–	–	–
B	0(d)	0(d)	0(d)	100(a)	100(a)	100(a)
Campyloneurum phyllitidis						
A	9(b)	40(a)	25(b)	74(a)	38(b)	56(a)
AA	48(a)	61(a)	55(a)	–	–	–
AB	55(a)	64(a)	59(a)	–	–	–
BB	49(a)	65(a)	57(a)	–	–	–
B	5(b)	61(a)	33(b)	83(a)	31(b)	57(a)
Phlebodium aureum						
A	77(b)	82(ab)	79(b)	18(a)	13(a)	16(a)
AA	92(ab)	90(ab)	91(ab)	–	–	–
AB	98(ab)	80(ab)	88(ab)	–	–	–
BB	89(ab)	100(a)	94(a)	–	–	–
B	92(ab)	97(ab)	95(a)	7(a)	3(a)	5(a)
Microgramma heterophylla						
A	0(b)	2(b)	1(b)	100(a)	98(a)	99
AA	32(a)	24(a)	28(a)	–	–	–
Phymatosorus scolopendria						
A	88(a)	90(a)	89(a)	12(a)	10(a)	11
AA	93(a)	93(a)	93(a)	–	–	–
Polypodium pellucidum						
A	0(c)	2(c)	1(b)	100(a)	95(a)	98
AA	63(a)	26(b)	43(a)	–	–	–

[1] A and B indicate single gametophyte cultures; AA, AB and BB indicate paired cultures, AA and BB indicate gametophyte pairs from the same sporophyte, AB indicates gametophyte pairs from two different sporophytes.

[2] Genetic load was estimated by counting the percentage of bisexual gametophytes failing to produce sporophytes.

[3] The same letter in parentheses for each item for each species indicates no significant difference in Duncan's multiple test (95% confidence limits).

paired cultures. However, single plants isolated as spores produced significantly fewer sporophytes than those isolated as young gametophytes or those grown in pairs. Thus genetic load estimates from isolated-spore and isolated-gametophyte cultures were significantly different (Table 2).

In *P. pellucidum*, 21% of the isolated plants produced sporophytes. However, only 1% of these were produced through syngamy, the others were generated through apogamy. All of the apogamous sporophytes formed from the tips of their parent gametophytes from vegetative tissue. None of these apogamous sporophytes grew as well as sexually produced ones. They remained dwarfed, turned yellow-white, and died eventually.

TABLE 3. Allele frequencies at polymorphic loci in three populations of *Campyloneurum phyllitidis*. Sample sizes in each population are 10. Populations were invariable at 27 loci.

Locus	Allele	JD[1]	CH[1]	FS[1]	Locus	Allele	JD[1]	CH[1]	FS[1]
Lap-b	2	1.00	0.10	0.65	Pgm-3a	1	1.00	1.00	0.90
	3	–	0.90	0.35		2	–	–	0.10
Mdh-3a	1	–	0.10	–	Pgm-3b	1	1.00	1.00	0.80
	2	1.00	0.90	1.00		2	–	–	0.20
Mdh-3b	1	–	0.10	–	6Pgd-2a	1	1.00	1.00	0.40
	2	1.00	0.90	1.00		2	–	–	0.60

[1] JD = Jonathan Dickson State Park, CH = Castellow Hammock, FS = Fakahatchee Strand State Preserve.

ISOZYMES.—In *C. phyllitidis*, seventeen putative locus pairs were scored across the eleven enzyme systems (Table 3). There was no variability within or among the three populations for 12. These were fixed for the same allele at four (Fbp-1, Idh, Mdh-1, and Tpi-1) and for fixed interlocus heterozygosity at eight locus-pairs (Aco, Fbp-2, Hk, Mdh-2, 6Pgd-1, Pgi-2, Skdh, Tpi-2). Mdh-3 was fixed for a single allele in the samples from JD and FS populations, Pgm-2 was fixed in all populations, but for a different allele at 2b in JD, and Pgm-3 was fixed in the samples from JD and CH populations. 6Pgd-2 had fixed heterozygosity in populations JD and CH. The genetic similarity among the three populations was high (>0.934) for both Rogers' (1972) and Nei's (1978) genetic coefficient (Table 4).

In population JD, only one genotype was found at each of the locus-pairs. In population CH, Lap and Mdh-3 each had two genotypes, combining to form three multilocus genotypes. In population FS, Lap and Pgm-3 each contained three genotypes and the variable locus 6Pgd-2 contained two genotypes, combining to form six multilocus genotypes (Table 5). In combination, the three populations form 10 multilocus genotypes. Of 30 plants tested, only one displayed recombinational heterozygosity at one locus (Lap 11/23).

In *P. aureum*, 22 putative loci were scored among the 13 enzyme systems. There was no variability within or among the three populations for 21 locus pairs. Eleven (Ald, Fbp-1, Fbp-2, Idh-1, Idh-3, Mdh-1, Mdh-3, 6Pgd-2, Pgi-1, Skdh-1, and Skdh-2) were fixed at the same allele, and ten (Aco-1, Aco-2, Hk, Lap, Mdh-2, 6Pgd-3, Pgi-2, Pgm-1, Pgm-2, and Tpi-2) had fixed heterozygos-

TABLE 4. Matrix of Roger's genetic similarity (above diagonal) and Nei's unbiased genetic identity (below diagonal) among three populations of *Campyloneurum phyllitidis* in Florida.

Population[1]	JD	CH	FS
JD	**	0.938	0.934
CH	0.946	**	0.951
FS	0.956	0.981	**

[1] JD = Jonathan Dickson State Park, CH = Castellow Hammock, FS = Fakahatchee Strand State Preserve.

TABLE 5. Description of multilocus genotypes of two populations of *Campyloneurum phyllitidis* in Florida.

Locus	CH[1]			FS[1]					
Lap-a/b	11/22	11/33	11/33	11/22	11/22	11/33	11/33	11/23	11/22
Mdh-3a/b	22/22	11/11	22/22						
Pgm-3a/b				11/11	11/22	11/11	22/22	11/11	11/11
6Pgd-2a/b				11/22	22/22	11/22	11/22	22/22	22/22
Observed	10%	10%	80%	30%	10%	20%	10%	10%	20%

[1] CH = Castellow Hammock, FS = Fakahatchee Strand State Preserve.

ity. Got displayed fixed heterozygosity for the same alleles in TS and JD populations and in 90% of the samples in FS, but had fixed heterozygosity with a different allele for Got-b in the other 10%. The genetic similarity between population FS and the two identical populations TS and JD was high, 0.998 in Rogers' (1972) genetic similarity and 1.0 in Nei's (1978) genetic identity. In populations TS and JD, there was only one genotype for all the locus-pairs. In population FS, only two genotypes appeared, Got-a^{11}/b^{11} (10%) and Got-a^{11}/b^{22} (90%). No recombinational heterozygous individuals were detected among the 30 plants tested.

DISCUSSION

In multi-gametophyte cultures of all species, male and bisexual plants appeared only after a significant number of female plants had differentiated. This is consistent with the presence of antheridiogen systems in these species, as has been previously demonstrated (Chiou & Farrar, 1997a). Antheridia on bisexual gametophytes grown as isolated plants were probably induced by their own gametophyte's antheridiogen secretions. This could occur either by absence of or delayed attainment of insensitivity of a gametophyte to antheridiogen, or by generation of secondary lobes with reduced physiological connection to the principal antheridiogen-producing apex. A small number of males among plants isolated as spores in most species suggests the further possibility that some antheridia may form without stimulation from antheridiogen. Plants that remained unisexual males at eight months generally were relatively slow-growing and small.

In isolated-plant cultures, a significant difference in sexual expression between gametophytes grown from isolated spores and those grown from gametophytes isolated from multi-gametophyte cultures when they were one month old was found only in *Campyloneurum phyllitidis* and *Polypodium pellucidum*. In these two species, bisexual gametophytes were much more abundant in isolated-gametophyte cultures than in isolated-spore cultures, whereas unisexual females were more abundant in isolated-spore cultures. It is likely that the difference between culture types of *C. phyllitidis* and *P. pellucidum* is that plants in multi-gametophyte cultures were subjected to antheridiogen before individual gametophytes were transferred to the iso-

lated-gametophyte cultures. It is also possible that transplanting actively growing gametophytes might disrupt their normal developmental pattern. Neither hypothesis explains the slow rate at which bisexuality is attained by spore-isolated gametophytes of these species relative to the other species tested.

Genetic load can be estimated by the ability of isolated bisexual plants to produce sporophytes. Isolated gametophytes of *Polypodium pellucidum*, *Microgramma heterophylla*, and the B source of *Campyloneurum angustifolium* produced virtually no sporophytes through syngamy, whereas isolated gametopytes of *C. phyllitidis*, *Phlebodium aureum*, *Phymatosorus scolopendira* and the A source of *C. angustifolium* produced abundant sporophytes through intragametophytic selfing.

Genetic load was not significantly different between isolated-spore and isolated-gametophyte cultures, except in *Campyloneurum phyllitidis* where the apparent genetic load of isolated-spore cultures was much greater. The reason for this apparent elevation of genetic load in isolated-spore cultures in *C. phyllitidis* is not clear, but from the delayed production of bisexual plants evident in isolated spore cultures (Table 1), it is possible that antheridia in some bisexual plants may still have been functionally immature even at eight months, falsely indicating a high genetic load. No similar delay in production of antheridia by this species was evident in multispore cultures (Chiou & Farrar, 1997a) or in isolated-gametophyte cultures. However, pairing of gametophytes from different sporophytes in *C. phyllitidis* also failed to fully relieve the sporophyte suppression observed in isolated gametophyte cultures. This suggests that some genetic load in this species is perhaps being expressed in gamete development (Klekowski, 1971), or that there was very little genetic difference between the two sporophytes, as might well be the case in a population reproducing primarily by intragametophytic selfing. In fact, most sporophytes of this species were homozygous at all loci tested.

Paired-spore and paired-gametophyte cultures allowed intergametophytic mating to relieve inbreeding depression that might prevent intragametophytic selfing in either gametophyte. Thus any increase in sporophyte production in paired cultures relative to isolated cultures of the same species is assumed to have resulted from intergametophytic mating.

Two spore sources were used in studies of *Campyloneurum angustifolium*, *C. phyllitidis*, and *Phlebodium aureum*. Genetic load estimates obtained from the two sources were not significantly different for *C. phyllitidis* and *P. aureum*. Estimated genetic load did differ significantly between the two sources of *C. angustifolium* in which it was low for source A, but very high for source B. This suggests that the B sporophyte of *C. angustifolium* was from a highly outcrossing population, whereas the A sporophyte was derived from a population with a higher level of inbreeding. Both diploid and tetraploid chromosome counts have been reported for *C. angustifolium* (n = 37, Evans, 1963, from Peru; n = 74, Evans, 1963, from Costa Rica; Sorsa, 1966, from Costa Rica; Knobloch, 1967, from Jamaica). Thus, judging from a correlation

TABLE 6. Description of multilocus genotypes and their percentage in a combination of three Florida populations of *Campyloneurum phyllitidis*.

Lap-a/b	Mdh-3a/b	Pgm-2a/b	Pgm-3a/b	6Pgd-2a/b	Percentage
11/22	22/22	11/22	11/11	11/22	33.33
11/33	22/22	11/11	11/11	11/22	26.67
11/33	11/11	11/11	11/11	11/22	3.33
11/22	22/22	11/11	11/11	11/22	3.33
11/33	22/22	11/11	11/11	11/22	6.67
11/22	22/22	11/11	11/11	11/22	10.00
11/23	22/22	11/11	11/11	22/22	3.33
11/33	22/22	11/11	22/22	11/22	3.33
11/22	22/22	11/11	11/22	22/22	3.33
11/22	22/22	11/11	11/11	22/22	6.67

of polyploidy with low genetic load, it is possible that the A sporophyte and B sporophyte of *C. angustifolium* were tetraploid and diploid respectively.

Both diploid and tetraploid forms have also been reported in *Phlebodium aureum* (n = 37, n = 74, Evans, 1963), and *Phymatosorus scolopendria* (2n = 72, Löve et al., 1977; n = 72, Tsai and Shieh, 1983). In *Campyloneurum phyllitidis*, only tetraploids have been found (n = 74, 2n = 148, Evans 1963, Nauman 1993), whereas in *Polypodium pellucidum*, only diploid numbers have been reported (Manton, 1951). Thus genetic load estimates indicate that the sample sporophyte spore sources of *C. phyllitidis*, *P. aureum* and *P. scolopendria* were probably tetraploids, whereas those of *Microgramma heterophylla*, and *P. pellucidum* were diploid. In fact, isozyme evidence also indicates that *C. phyllitidis* and *P. aureum* are polyploids.

Strong evidence for describing the mating system of diploid species in the wild can be obtained from analysis of isozyme electrophoretic patterns. Electrophoretic patterns for the species tested in this study, *Campyloneurum phyllitidis* and *Phlebodium aureum*, revealed a high level of fixed heterozygosity for both, indicating that these samples from Florida are polyploid, probably allopolyploid. Because of this, accurate counts of heterozygous individuals and estimates of outcrossing from isozyme evidence are not possible, although we can state that at least one putatively outcrossed individual (Lap-a^{11}/b^{23}) was among the 10 samples of *C. phyllitidis* in the FS population. No evidence of outcrossing was present in the Florida populations of *P. aureum* but the extremely low level of genetic variability among sampled plants of *P. aureum* (29 of 30 plants were genetically identical) would preclude isozymic detection of most recombinational heterozygotes if they were formed. However, the considerable variability among sporophytes of *C. phyllitidis* (10 multilocus genotypes among 30 plants tested) allows ample opportunity for detection of unbalanced heterozygotes and three-allele heterozygotes (at Lap) that would be produced if outcrossing was frequent (Table 6).

Assuming allopolyploidy, intragametophytic selfing, and no mutations, the maximum number of genotypes per locus-pair provides an estimate of the

minimum number of hybridization events involved in producing an allotetraploid species (Ranker et al., 1994). Thus in *Campyloneurum phyllitidis*, the single genotype in the population JD suggests that only one hybridization event occurred in the ancestry of this population in Jonathan Dickson State Park. Two genotypes at each of Lap and Mdh-3 of population CH indicate that that population originated from at least two hybridization events. Three genotypes at Pgm-3a/b implies that at least three hybridization events produced population FS. However, one of these genotypes (11/22) could have been generated (11/11 × 22/22) by the low level of outcrossing demonstrated by presence of the Lap 11/23 genotype. Because there are not great distances separating these populations in Florida and because of the high similarity of genotypes among the three populations, we can also consider them as a single population. In that case the number of hybridization events responsible for the Florida plants is at least three and may be as high as ten.

In *Phlebodium aureum*, the genetic similarity among sampled populations is very high. Isozyme evidence showed no genetic variation in the samples of TS and JD populations and only a single plant representing a distinct genotype in the FS population. Because *Campyloneurum phyllitidis* and *P. aureum* are widespread in tropical America and because likely parental diploids are not present in Florida, these Florida genotypes probably represent three to ten separate spore introductions for *C. phyllitidis* and possibly only one or two for *P. aureum*.

In an electrophoretic study of *Polypodium pellucidum*, Li and Haufler (1999) found a small but significant (mean fixation index of 0.169 across populations of epiphytic *P. pellucidum* var. *pellucidum*) excess of homozygous individuals in most (but not all) populations sampled in the Hawaiian archipelago. Since intragametophytic selfing as a dominant breeding system would lead to much higher fixation values in relatively few generations (Hartl & Clark, 1997), the observed level of fixation likely results from a mixed mating system where intergametophytic selfing among gametophytes in populations derived from the same sporophyte is occurring. Thus electrophoretic analysis of population structure in *P. pellucidum* is not inconsistent with the implications of our results that intragametophytic selfing in this species is rare, probably curtailed by genetic load. This constraint on reproduction via single isolated spores likely contributes to the genetic differentiation and low values of gene flow between populations estimated by Li and Haufler (1999).

The fact that diploidy favors intergametophytic mating whereas tetraploidy favors intragametophytic selfing has been demonstrated (e.g., Masuyama and Watano, 1990). The fixation of different alleles from the different parent species of allopolyploids may mitigate the expression of recessive lethal genes caused by intragametophytic selfing. In our study, low levels of genetic load, as evidenced by production of sporophytes through intragametophytic selfing, was well correlated with polyploidy (Table 7). Isolated gametophytes of the diploid species (*Polypodium pellucidum*, *Microgramma heterophylla*, and the B source of *Campyloneurum angustifolium*) produced virtually no

TABLE 7. Genetic load, ploidy level and probable mating systems in Polypodiaceae species.

Species	Genetic load[1]	Ploidy[2]	Mating system[3]
C. angustifolium A[4]	14	P (Evans 1963)	Intragam. selfing
C. angustifolium B[4]	100	D (Evans 1963)	Intergam. mating
C. phyllitidis	35	P (Isozyme; Evans 1963)	Intergam. selfing
M. heterophylla	99	D	Intergam. mating
Phl. aureum	10	P (Isozyme; Evans 1963)	Intergam. selfing
Phy. scolopendria	11	P (Tsai & Shieh 1983)	Intergam. selfing
Pol. pellucidum	98	D (Manton 1951)	Intergam. mating

[1] Estimated from average sporophyte production by single gametophytes isolated as spores and as young gametophytes, except for *C. phyllitidis* in which the estimate is from plants isolated as young gametophytes.
[2] Determined from isozyme patterns (isozyme) and/or chromosome counts (reference). D = diploid, P = polyploid.
[3] Determined from isozymes and/or genetic load.
[4] *C. angustifolium* A and B plants are from two different sources.

sporophytes through syngamy, whereas isolated gametopytes of the tetraploid species (*C. phyllitidis*, *Phlebodium aureum*, *Phymatosorus scolopendria* and the A source of *C. angustifolium*) produced abundant sporophytes through intragametophytic selfing.

Gametophytes derived from isolated spores resulting from long-distance spore dispersal are likely to be isolated from other gametophytes of the species. Such gametophytes are capable of producing sporophytes through intragametophytic selfing, provided they have low genetic load and generate bisexual gametophytes. Gametophytes in populations developed from many spores can produce sporophytes through either intragametophytic selfing or intergametophytic mating, the latter being augmented by antheridiogen-stimulated production of male gametophytes. Species predominantly reproducing by intergametophytic mating can maintain high levels of genetic variability, including a high genetic load, but may be very limited in their ability to migrate by long-distance spore dispersal (Peck et al., 1990). Thus a trade-off exists between maintenance of genetic variability on one hand and ease of migration on the other.

Schneller et al. (1990) concluded that a correlation exists between antheridiogen response and genetic load. The antheridiogen systems (Chiou & Farrar, 1997a) of *Microgramma heterophylla* and *Polypodium pellucidum* are consistent with this correlation, but the presence of antheridiogen systems in *Campyloneurum angustifolium* (source A), *C. phyllitidis*, *Phlebodium aureum* and *Phymatosorus scolopendria* (Chiou & Farrar, 1997a) is contradictory. If the low genetic loads of the latter group indicate their polyploid status (and isozyme patterns of fixed heterozygosity also indicate that *C. phyllitidis* and *P. aureum* are polyploid), their maintenance of antheridiogen systems must be explained. Possibly antheridiogen production and response is a vestige from their diploid ancestors and of no significance to the breeding system of the polyploid species (Haufler and Gastony, 1978;

Schneller & Hess, 1995). But, the existence of antheridiogens here could also function to promote bisexuality in isolated plants if individual thalli have reduced ability to attain bisexuality. Gametophytes of these species propagate vegetatively by branching (Chiou & Farrar, 1997b). Maintaining an antheridiogen system may be adaptive in promoting antheridium formation on new vegetatively produced thalli regardless of whether the species is inbreeding or outbreeding.

Whether species are outbreeders or inbreeders, breeding behavior must be adaptive to establishment and survival of individuals of those species. In general, inbreeding may be advantageous for initiating new populations following long-range spore dispersal where gametophytes are likely to be derived from single isolated spores. The opposite strategy, outbreeding, has the advantage of generating and retaining genetic diversity, and high genetic loads carried by outbreeders tend to maintain that mating system. Previous studies have demonstrated that gametophytes of the epiphytic species studied here grow perennially through branching and vegetative proliferations which increase their life span and effective gametophyte size (Chiou & Farrar, 1997b) and produce antheridiogen that facilitates the production of male gametophytes (Chiou & Farrar, 1997a). Both of these characteristics have been proposed to increase the probability of intergametophytic mating (Chiou & Farrar, 1997a; b; Chiou et al., 1998). Here we demonstrate that sporophytes of these species may be produced through either outcrossing or inbreeding, as evidenced by high or low level of genetic load, respectively. Their mating systems may be controlled principally by genetic load and generally correlated with ploidy level. Interwoven with these factors are gametophyte morphology, growth habit, antheridiogen production, and environmental parameters which together maintain successful reproduction of these species. For outbreeding diploid species, genetic load is possibly the driving force leading to morphological and physiological adaptations promoting outcrossing.

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Belowground Distribution and Abundance of *Botrychium* Gametophytes and Juvenile Sporophytes

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ABSTRACT.—A significant portion of the stages of the life history of *Botrychium*, the gametophyte and juvenile sporophytes, are spent belowground. Surveys were conducted to determine the distribution and abundance of belowground gametophytes, juvenile sporophytes and gemmae of eight species of *Botrychium*. For each species, soil samples were collected in a 200 m^2 area, sifted through a series of soil sieves, and centrifuged to separate the lighter plant material. Only 40% of the soil samples contained belowground structures revealing a patchy distribution. The gametophytes of *B. montanum* are most dense, followed *by B. mormo* with 738 and 728 gametophytes m^{-2} respectively. *Botrychium hesperium* also has a relatively high density of 478 gametophytes m^{-2}. *Botrychium gallicomontanum* is the least dense with 10 gametophytes m^{-2}. *Botrychium campestre and B. gallicomontanum* both have abundant gemmae and few gametophytes. The density of individuals in the belowground structure bank greatly exceeds the aboveground population. The size and health of the belowground structure bank is critical in sustaining the long-term aboveground population and in buffering it from extinction.

The importance of propagule banks (also referred to as seed, spore, or diaspore banks) in community dynamics has long been recognized (Leck et al., 1989) for flowering plants. Propagules may persist belowground for many years, creating a secure reservoir from which aboveground plants can be reestablished following extinction during unfavorable environmental conditions. The propagule bank serves as a buffer against extinction during unfavorable environmental conditions as a reservoir of genes, and as such is an important factor in determining community dynamics.

Relatively few studies have documented the propagule banks of ferns. In previous studies, soil has been collected, and spread in containers in glasshouses to cultivate spores that may be present in the propagule bank (Dyer and Lindsay, 1992; During and ter Horst, 1983; Hamilton, 1988; Milberg, 1991; Schneller, 1988). Three major trends have been noted. First, the density of fern gametophytes resulting from cultivation of spore banks is high, ranging from 57,000 spores m^{-2} (Milberg, 1991) to 5,000,000 m^{-2} (Schneller, 1988). Second, the abundance of fern spores in the spore bank is a result of the longevity of spores and the long-term accumulation of spores (Dyer, 1994; Milberg, 1991). Third, when comparing the belowground density

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of propagules (represented by germinated gametophytes) to the aboveground density of sporophytes, all previous studies have reported the remarkable lack of ferns present in the aboveground vegetation despite a belowground reservoir of spores. During and ter Horst (1983), Leck and Simpson (1987), Milberg (1991), Raffaele (1996), Rydgren and Hestmark (1997) and Strickler and Edgerton (1976) all found evidence of high densities of fern propagules where ferns were entirely absent from the aboveground vegetation.

Botrychium presents two problems in applying conventional propagule bank techniques. First is the difficulty and length of time required to culture *Botrychium* beyond germination (Whittier and Thomas, 1993). *Botrychium* spores require darkness for germination, and in nature *Botrychium* gametophytes require mycorrhizae for growth beyond the two- or three-celled stage. Therefore, it is not possible to culture and quantify the propagules using standard seed bank techniques.

The second and more significant problem relates to the different life cycle of *Botrychium*, relatively little of which is visible aboveground (Fig. 1). Plants produce spores that filter into the soil and germinate in darkness. Following germination, a belowground achlorophyllous, fleshy gametophyte is produced. The gametophyte produces gametangia and sexual reproduction occurs, resulting in a belowground juvenile sporophyte. The belowground rhizome is upright and short with mycorrhizal roots (no root hairs) and a single leaf-producing bud at the apex. It takes several years for this juvenile sporophyte to produce a leaf-bearing apex and emerge aboveground (Johnson-Groh et al., 1998). The plants generally produce one leaf annually, but it is common for *Botrychium* plants to remain dormant belowground in a given year and produce no aboveground leaf (Johnson-Groh, 1997).

In addition to these belowground stages, some species reproduce asexually via belowground gemmae, small (0.5–1 mm) propagules that can independently give rise to a new plant once detached from the parent plant (Farrar and Johnson-Groh, 1990). Gemmae form on the rhizome and abscise at maturity. Upon germination, gemmae develop 4 or 5 short roots prior to the differentiation of a shoot apex and production of leaves (Farrar and Johnson-Groh, 1990). The first leaves formed are short and slender and do not reach the soil surface. The presence of vegetative reproduction greatly influences the population dynamics of these gemmiferous species. It is common in the field to see two or more leaves of gemmiferous *Botrychium* emerging in close proximity. Excavation of these clusters usually reveals a large number of belowground sporophytes in various stages of development.

The terms propagule bank and diaspore bank as used in other studies imply banks of structures that have been disseminated (e.g. seeds, spores). Except for gemmae, *Botrychium* gametophytes and sporophytes are not structures designed specifically to propagate or disseminate. Consequently the use of existing terms (propagule, diaspore, spore or seed bank) does not accurately describe the belowground structures of *Botrychium*. The term belowground structure bank, as used in this paper, includes all belowground structures: gemmae, gametophytes, juvenile plants and spores.

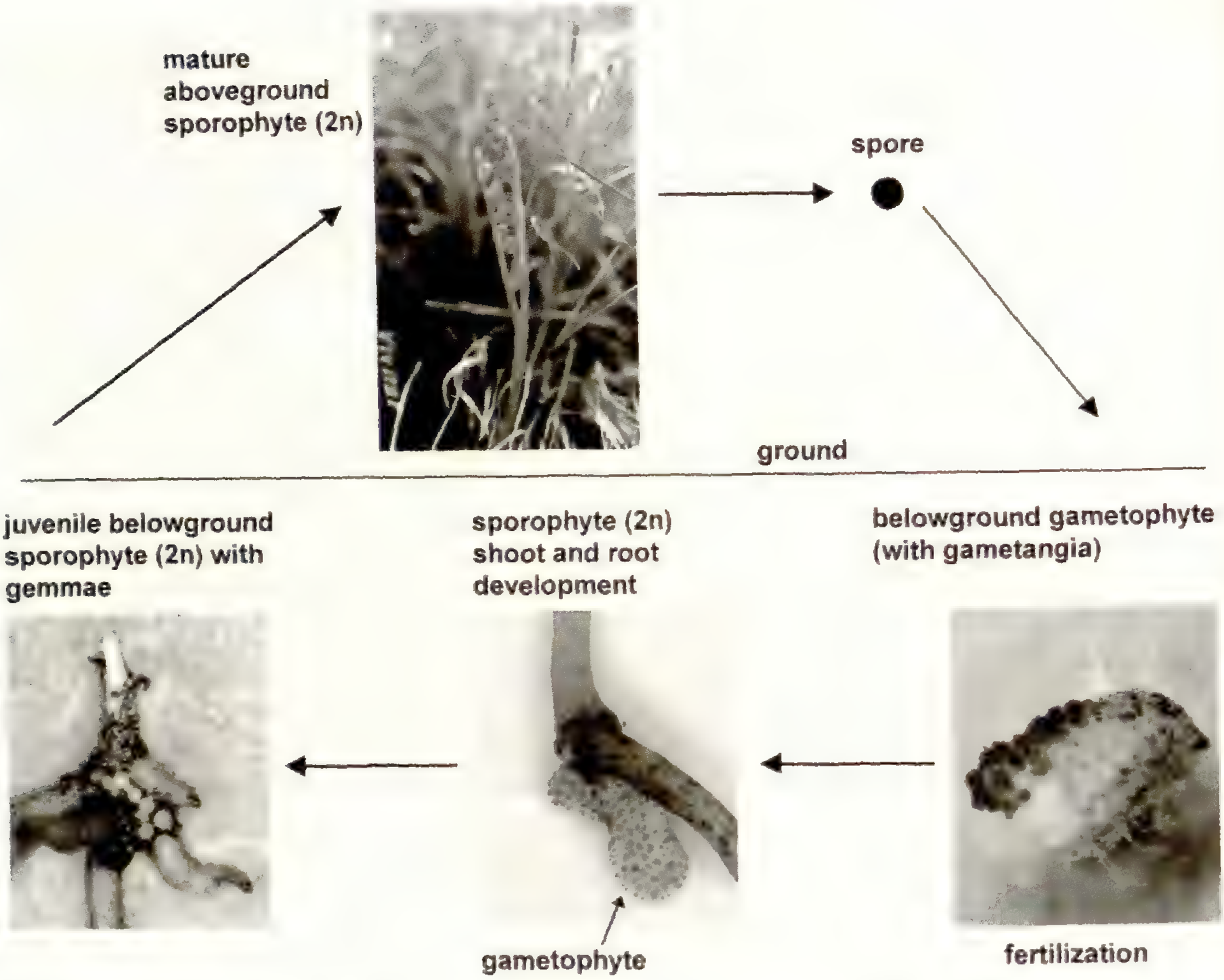

FIG. 1. *Botrychium* life cycle illustrating the belowground developmental stages.

Long-term demographic studies (15 years) of *Botrychium* reveal that population numbers are quite variable (Johnson-Groh, 1997). Aboveground *Botrychium* population numbers fluctuate independently within and between populations, as well as between years and between different sites. Fire, herbivory, herbicides, and timber harvests may have an immediate impact on the aboveground sporophytes (Johnson-Groh and Farrar, 1996). However, the aboveground populations are fairly resilient and rebound following perturbations, although recovery may take several years.

Many species of *Botrychium* are considered rare. Several are listed as critical, threatened, or endangered (Minnesota Department of Natural Resources, 2002). Understanding *Botrychium* population dynamics, including their belowground biology, is necessary to effectively manage these species. A more complete understanding of the belowground structure bank will allow prediction of the impact of various management regimes, such as fire or grazing, on these rare species.

The goal of this research was to investigate the belowground structure bank of several species of *Botrychium*. It seems likely that the belowground structures (gametophytes, juvenile sporophytes, gemmae and spores) play a

key role in the population dynamics of *Botrychium*, and it is anticipated that the number of belowground structures should be at least as many as the number of aboveground plants.

Materials and Methods

Sites for sampling the belowground populations of *Botrychium* were selected in areas that contained a typical density of aboveground plants (~50–400 plants in an area 200 m^2). A sampling grid resembling spokes of a wheel was established within each population, and samples were collected with a bulb planter at one-meter intervals along the six 8-meter spokes (Fig. 2). In addition to the 48 spoke samples, a sample was collected from the middle. A bulb planter (5-cm diameter) assured a uniform sample volume of approximately 200 cm^3 for each sample. Distance from each sample to the nearest aboveground plant, aboveground population density, and notes on the vegetation and general ecology were recorded.

Soil samples were collected during the summers of 1988 to 1999 from a variety of sites; these were processed the following academic years (Table 1). Seven species of *Botrychium* subg. *Botrychium* were investigated: *Botrychium campestre* W. H. Wagner & Farrar, *B. hesperium* (Maxon & R. T. Clausen) W. H. Wagner & Lellinger, *B. gallicomontanum* Farrar & Johnson-Groh, *B. lanceolatum* (S. G. Gmel.) Ångstr., *B. montanum* W. H. Wagner, *B. mormo* W. H. Wagner, *B. yaaxudakeit* Stensvold & Farrar, *B. virginianum* (L.) Sw. An eighth species, *Botrychium virginianum* (L.) Sw. from subg. *Osmundopteris*, was surveyed from two different sites. Because *Botrychium* typically grows in mixed species assemblages, it is difficult to locate populations of only one species. The sites sampled for *B. campestre*, *B. hesperium*, and *B. virginianum* consisted only of those species, respectively. All other sites contained more than one species. In all cases, the named species was the dominant species (>75% of aboveground *Botrychium*).

Soil samples were processed using centrifugation, which allows the lighter plant material to be extracted for examination under the microscope (Mason and Farrar, 1989). The soil sample was broken up and washed through a series of soil sieves where larger roots and debris were removed. The sediment was collected and root segments dyed in neutral red and cut to the approximate size of *Botrychium* gemmae (0.5 mm) were added to the sediment to gauge the success of the procedure. Samples were sieved in successively finer sieves and then centrifuged first in water and then in sucrose solutions. Centrifugation separated the belowground structures from the denser soil particles. The first centrifugation caused dead organic matter to float to the top of the tube, leaving living organic matter and stained roots in the pellet. A second centrifugation in sucrose caused the living material to float.

The decanted liquid containing *Botrychium* plants was examined under the microscope for gemmae, gametophytes, sporophytes, and stained root segments. Gametophytes were usually small (<1 mm) and irregularly shaped, and

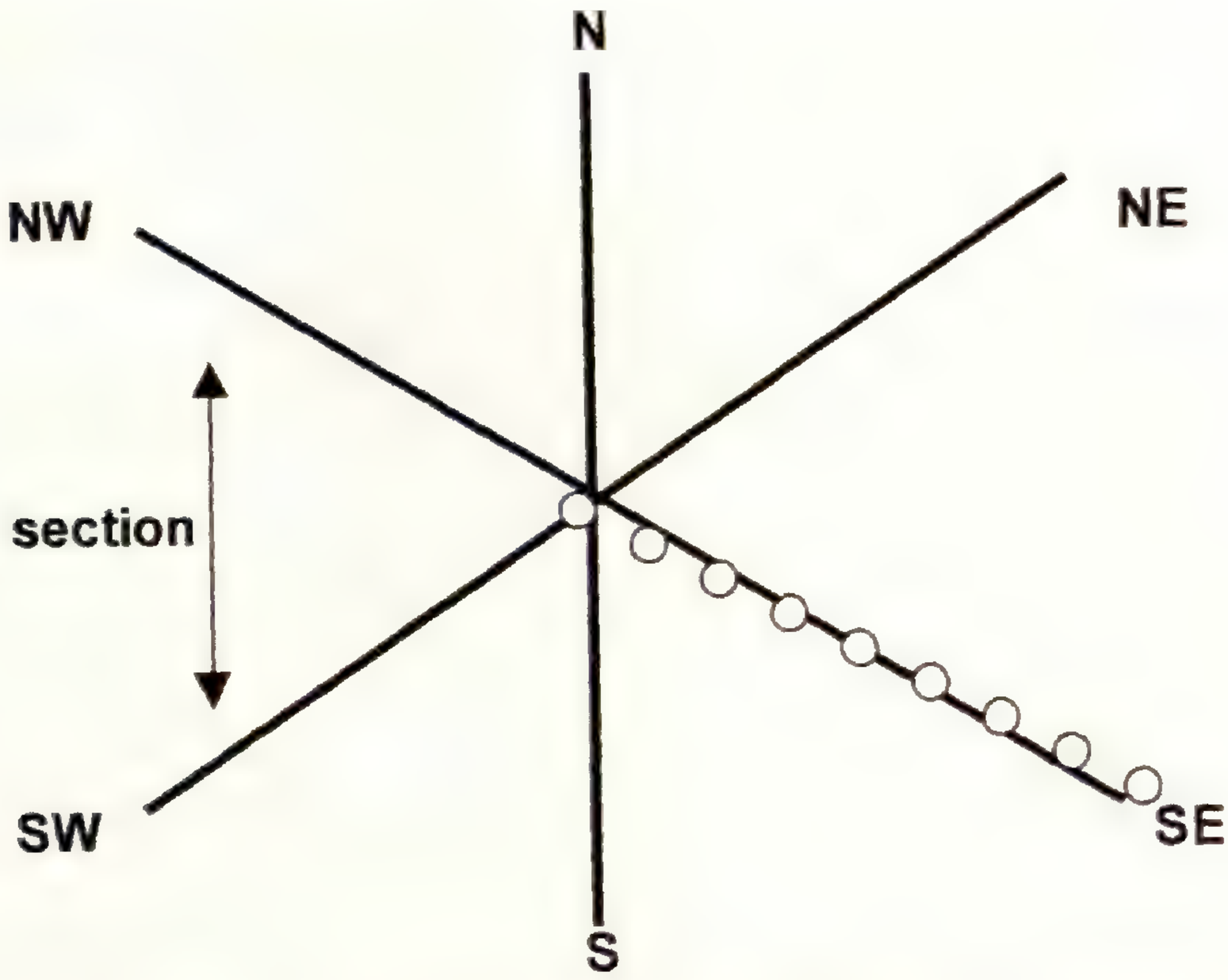

FIG. 2. Sampling design for collecting 49 soil samples for analysis. Circles represent soil samples collected on one of 6 radii.

could be identified by the presence of rhizoids, antheridia, and archegonia. Juvenile sporophytes represented a continuum of development following the formation of the embryo (still attached to the gametophyte) through the development of a mature plant. Except for very young sporophytes, all juveniles had roots that were succulent and lacked root hairs. Very young sporophytes, here referred to as embryos, were small (<1 mm), spherical to irregular in shape, lacked rhizoids, and, when detached from the gametophyte, had a relatively large scar where they had been attached. Gemmae likewise represented a continuum of development following their formation. At abscission, gemmae were spherical and small (0.5–1 mm). As gemmae began to elongate and form roots, it was impossible to distinguish them from juvenile sporophytes originating from a gametophyte. Examination of rhizomes of mature leaf-bearing plants determined which species regularly produce gemmae.

RESULTS AND DISCUSSION

Results of the belowground analysis are shown in Table 2. The density of aboveground sporophytes ranged from 0.4 m^{-2} for *B. hesperium* and *B. virginianum* to 16.1 m^{-2} for *B. gallicomontanum*. The proportion of samples that contained belowground structures ranged from 4% for *B. campestre* to 65% for *B. hesperium*, with an overall average of 30%. *Botrychium campestre* and *B. virginianum*, had notably low frequencies. The density of belowground gametophytes ranged from 10 m^{-2} for *B. gallicomontanum* and one

TABLE 1. *Botrychium* species surveyed for belowground analysis, their sampling location, and year of collection.

Species	Location	Year
B. campestre	IA, Plymouth County, 5-Ridge Prairie	1988
B. hesperium	WA, Colville Nat. Forest, Big Muddy	1998
B. gallicomontanum	MN, Norman County, Frenchman's Bluff	1995
B. lanceolatum	OR, Wallowa-Whitman Nat. Forest, Lostine Creek	1999
B. montanum	WA, Colville Nat. Forest, Kerry Creek Seep	1998
B. mormo	MN, Chippewa Nat. Forest, Ottertail Peninsula	1995
B. yaaxudakeit	AK, Tongass Nat. Forest, Yakutat	1999
B. virginianum (So. MN)	MN, Nicolett County, St. Peter	1997
B. virginianum (No. MN)	MN, Superior Nat. Forest, Pike Mountain	1999

population of *B. virginianum* to 738 m^{-2} for *B. montanum*. The density of belowground juvenile sporophytes ranged from 0 m^{-2} (*B. gallicomontanum*, *B. montanum*, and *B. virginianum*) to 281 m^{-2} (*B. hesperium*). The average stained root recovery was 84%.

Three of the surveyed species are known to produce gemmae (*B. campestre*, *B. hesperium*, and *B. gallicomontanum*). For those species that produce gemmae, the density ranged from 5,907 gemmae m^{-2} for *B. campestre* to 21 m^{-2} for *B. hesperium* (Table 3). Gemmae, or gemma-like structures, were also found in samples in which the dominant species does not produce gemmae. For example, *B. lanceolatum* does not produce gemmae, but gemmae were found in the samples. Two associated species, *B. minganense* and *B. pedunculosum*, were also found at the *B. lanceolatum* site and are known to produce sparse gemmae, so it is possible that the gemmae extracted belong to these associated species. (It is impossible to distinguish belowground structures of species except sometimes through absence or presence of gemmae.) *Botrychium yaaxudakeit* also does not produce gemmae, and it is likely that the gemmae found in this survey were from *B. ascendens*, which produces numerous gemmae, or even *B. minganense*, which produces few gemmae.

The high number of gemmae in *B. lanceolatum* may also be due to a peculiar behavior observed in this species of expelling the embryo from the gametophyte. Embryos appear to be "ejected" through the degeneration of surrounding gametophyte tissue at a stage very similar in size and morphology to gemmae produced in other species. This behavior may allow production of multiple embryos per gametophyte, increasing the total production of sporophytes. Further investigations of this behavior are underway.

There is large variation (4–65%) among species with regard to their frequency in the soil samples. Whether this is due to natural variation among species in response to environmental differences (e.g., *Botrychium campestre* may naturally have a lower frequency than *B. hesperium*) or to chance is not easy to resolve. Two technical problems prohibit easy resolution of this. First, centrifugation and microscopic techniques used for this investigation

TABLE 2. Result of *Botrychium* belowground analysis, with the maximum and minimum values for each column in boldface.

Species	Density of aboveground sporophytes (m^{-2})	Frequency of belowground structures (%)	Density of gametophytes (m^{-2})	Density of belowground juvenile sporophytes (m^{-2})	Density of gemmae (m^{-2})	Total density of belowground structures (m^{-2})	Ratio of belowground to aboveground plants
B. campestre	6.7	4	21	198	5907	6126	914
B. hesperium	0.4	65	478	281	21	780	1950
B. gallicomontanum	16.1	27	10	0	4170	4180	260
B. lanceolatum	3.1	61	135	10	520[1]	665	215
B. montanum	1.2	41	738	0	0	738	615
B. mormo	12.8	46	728	104	0	832	65
B. yaaxudakeit	1.4	29	281	42	312[1]	635	454
B. virginianum (So. MN)	0.6	8	73	0	0	73	122
B. virginianum (No. MN)	0.4	6	10	0	0	10	25
Average (all)	4.7	30	261	71	1228[1]	1560	332
Average (moonworts)	6.0	40	324	91	1579[1]	1994	332

[1] See discussion for explanation of "gemmae" in soil samples for non-gemmiferous species.

TABLE 3. Soil surveys that contained gemmae produced either by the dominant primary species or by associated species.

Dominant species	Density of gemmae (m^2)	Dominant species known to produce gemmae	Associated species found at the sampling site and known to produce gemmae
B. campestre	5907	Yes	None
B. hesperium	21	Yes	None
B. gallicomontanum	4170	Yes	None
B. lanceolatum	520	No	*B. minganense*, *B. pedunculosum*
B. yaaxudakeit	312	No	*B. ascendens*, *B. minganense*

are extremely labor-intensive, thereby precluding multiple repetitions of the same species. Second, although the sampling technique used for this study is nominally disruptive, it is prudent to minimize excessive disturbance of rare *Botrychium* species. For the latter reason, *B. virginianum* (which is not rare) was included in this survey (despite its position in a different subgenus) and was sampled twice. These samples reveal a high degree of consistency, with nearly identical belowground structure banks, even though the sites surveyed were more than 300 miles apart.

It is also likely that the frequency differences among species are increased by the patchy distribution of *Botrychium* and the probability of sampling those patches. Distributions of plants result from factors of dispersal, survivorship, and habitat heterogeneity. Because the distribution of aboveground plants is patchy the same might be expected belowground. The random chance of sampling a dense patch could account for the variation between species.

SPORES.—Although it is difficult to determine the presence of *Botrychium* spores in the soil, other spore bank studies have shown high diversity and abundance of fern spores that persist in the soil for many years (Dyer, 1994; Milberg, 1991). It is reasonable to assume that there is a long-lived bank of *Botrychium* spores. This bank is in constant turnover, receiving a variable annual input of spores and losing spores to predation, loss of viability, and other environmental perturbations.

Annual spore production is the primary means of restocking the spore bank. Spore production, however, can be expected to vary from year to year, depending on the number of aboveground plants and their development in any given year. Johnson-Groh and Lee (in press) found that only 55% of *B. gallicomontanum* and 39% of *B. mormo* produced spores in 1996. The remainder of the plants senesced prior to spore production. Similar results have been found for other species (Cabin and Marshall, 2000; Houle, 1998; Kalisz, 1991; Raffaele, 1996). Houle (1998) found temporal differences in the seed rain for *Betula alleghaniensis* and seedling establishment, but a con-

stant seed bank. Houle noted that the uncoupling of the seed bank, rain, and seedling establishment further contributes to the spatiotemporal heterogeneity. It seems probable that similar complex spatiotemporal relationships influence *Botrychium* distribution.

Compounding the variable numbers of spores added annually to the propagule bank is the limited dispersal of spores. It is unknown how widespread moonwort spores disperse but based on the work of Peck et al. (1990) on *Botrychium virginianum* we can conclude that most spores disperse within 5 m or less. Dyer (1994) found that the largest spore banks occurred in samples taken immediately below ferns and that at a distance of 2 m away from the spore source, the spore bank was notably smaller. It seems likely that a few spores may become airborne and disperse farther. Because of the ability of *Botrychium* gametophytes to self-fertilize, it is reasonable to expect that a single spore is capable of dispersing and establishing a new population (Farrar, 1998).

Over time, a sizeable *Botrychium* spore bank is established in the soil. *Botrychium* spores likely remain viable for long periods of time, as do many other ferns (Lloyd and Klekowski, 1970; Miller, 1968; Sussman, 1965; Windham et al., 1986). These spores are probably dormant until conditions (moisture and mycorrhizae) are adequate, at which time many or all the spores in that localized area germinate and develop.

Juvenile Recruitment and Survivorship.—Unlike most other flowering plants or ferns, the juvenile sporophyte stages of *Botrychium* remain belowground for a number of years. The belowground recruitment of gametophytes and juvenile sporophytes therefore can be compared to seedling or sporeling recruitment aboveground for flowering plants and most ferns. As with other plants, juvenile mortality is probably significant for *Botrychium*. With one exception (*B. campestre*), the gametophyte density exceeds the juvenile sporophyte density (Table 2). Likewise, in most cases the belowground density exceeds the density of aboveground sporophytes. Mortality (defined as the proportional change between stages) is greatest (93% for all species) between the juvenile sporophyte stage and emergent sporophytes. There is an average of 73% mortality between the gametophyte and juvenile sporophyte stages. This high juvenile mortality is common among many plants. If similar high mortality (95%) occurs between the spore and gametophyte stages, we can predict an average minimum spore density of approximately 6,000 spores m^{-2}. *Botrychium montanum* and *B. mormo* have the highest predicted spore densities of 15,000 spores m^{-2}; *B. virginianum* and *B. gallicomontanum* have the lowest at 100 m^{-2}. This is considerably lower than 5,000,000 m^{-2} estimated by Hamilton (1988) for two species of *Athyrium* or even 57,000 m^{-2} estimated by Milberg (1991) for grassland soil. This estimate is also lower than the estimate of 100,000 spores m^{-2} made by Johnson-Groh et al. (1998) for *B. mormo*. Given the need for mycorrhizal infection following germination, mortality at this stage is probably very high, and it is reasonable to expect high spore densities within *Botrychium* populations.

Species with gemmae (*B. campestre*, *B. gallicomontanum*) have a higher total belowground density than those without gemmae. Like spores, gemmae, once detached from the parent plant, require mycorrhizae for further development. Farrar and Johnson-Groh (1990) found relatively few gemmae that contained mycorrhizae, which could explain the low number of developing gemmae relative to the number of gemmae produced. (Gemmae obtain mycelia through their connection with the parent rhizome; if unsuccessful, they remain dormant.) The primary role of gemmae may be to maintain the population in a microsite that has already proven successful. The frequent occurrence of multiple stems within a small-localized area (1–4 cm^2) suggests that gemmae are effective in local propagation. Dispersal beyond a short distance is limited, as evidenced by the low frequency of the highly gemmiferous species (*B. campestre*, *B. gallicomontanum*).

Species that produce profuse gemmae produce the lowest number of gametophytes (*B. campestre*, *B. gallicomontanum*). Gemmae, a form of asexual reproduction, produce essentially the same genetic product that a selfing gametophyte produces. The advantage of gemma production is the positioning for immediate success (mycorrhizae present). A greater reliance on reproduction via spores and gametophytes by most species and the higher disperability of spores undoubtedly accounts for the higher frequencies in soil samples of the non-gemmiferous species. The advantage of spore–gametophyte production allows dispersal to new sites, thereby insuring that "assets are diversified," which may provide a long-term advantage to the species. To draw further from investment analogies, gemmae are short-term investments with immediate returns, whereas spores are long-term investments with greater evolutionary payback.

Soil Heterogeneity.—Variations in microtopography, microclimate, parent material, mycorrhizae, and microorganisms all influence soil heterogeneity (Stark, 1994), creating a patchy environment. Because the spatial scale is very small, conditions merely a few centimeters away may not be sufficient to induce germination, thus creating a patchy distribution of plants.

Of these variable factors, mycorrhizae are probably the most important for *Botrychium*. Moonworts require endophytic mycorrhizae for gametophyte and sporophyte development and are dependent on mycorrhizae as a significant source of carbohydrate, minerals, and water. This observation is based on several peculiar behaviors. First, similar to orchids, moonworts do not emerge every year. They frequently fail to emerge for one to three consecutive years, with no subsequent decrease in size or other negative effects (Johnson-Groh, 1998). Second, "albino" botrychiums have been observed. Another indication that *Botrychium* depends relatively little on its own leaves for photosynthesis is the observation that these leaves frequently do not emerge above the litter. In fact only a small proportion of the total population of some species actually emerges from the litter (Johnson-Groh, 1998). Herbivory and loss of leaves through fire do not affect the size and vigor of plants in the subsequent year (Johnson-Groh, 1998). Finally, if roots and

leaves of juvenile plants are produced one per year, as in adults, 5–8 years may be required for development from gametophyte to a mature sporophyte with an emergent photosynthetic leaf (Johnson-Groh, 1998). Juvenile plants must rely on mycorrhizae for carbohydrates. Thus, although there has been no physiological studies to confirm this, it seems certain that moonworts (*Botrychium* subg. *Botrychium*) may depend largely on mycorrhizae for carbon from other plants, in addition to that produced by their own photosynthesis.

If photosynthesis is not critical for this subgenus and mycorrhizae are primarily responsible for overall energy budget, then understanding the belowground biology of *Botrychium* is imperative. Indeed, assumptions made about the population biology of other ferns may be irrelevant to *Botrychium*. Health of the mycorrhizal connection may determine juvenile recruitment and survivorship, and *Botrychium* populations may appear or disappear in accordance with mycorrhizal health.

Mycorrhizae play an important role in nutrient acquisition. This may be especially important for *Botrychium* because of the inability of its roots to forage. Root-foraging has been observed in flowering plants (Caldwell, 1994). It allows them to respond to small-scale nutrient patches. However, *Botrychium* roots are relatively few (5–30/plant), do not have root hairs, and do not appear to have the morphological plasticity to forage for small-scale patches of soil nutrients. Typically roots extend almost perfectly horizontally for their entire length (3–20 cm). Only occasionally are roots observed to abruptly bend in another direction. Tibbet (2000) argued that mycorrhizae are especially important for roots that do not have the morphological plasticity to respond to small-scale nutrient patches. Mycelia rapidly colonize patches of soil nutrients, making them ideal foraging instruments of the autotroph. In *Botrychium* it seems highly probable that its mycorrhizal mycelia are more important than root proliferation in nutrient acquisition.

Botrychium species that have high belowground densities generally have high aboveground population densities. *Botrychium campestre*, *B. gallicomontanum*, and *B. mormo* have the highest below- and aboveground densities. *Botrychium virginianum* has a relatively low below- and aboveground density. The ratio of belowground to aboveground plants ranges from 65:1 in *B. mormo* to 914:1 in the gemmiferous *B. campestre*.

For each species in this study, a relatively small volume of soil (962 cm^2) was sampled across a large spatial grid (201 m^2). Estimations of density are derived from these results. The patchy distribution and inability to sample large volumes of soil make it difficult to determine precise belowground populations. The opposite approach of intensively and completely surveying both above- and belowground in a small area is currently being investigated for *B. virginianum* and *B. campestre*. Preliminary results indicate that the belowground density in a small patch is several times the aboveground density.

Despite the difficulty of making direct comparisons below- and aboveground, in all cases it is readily apparent that belowground structures are much more abundant than aboveground plants. Sizeable reservoirs of

belowground structures are extremely important to the population dynamics of *Botrychium* and serve to replenish the populations following environmental perturbations. Because moonworts often remain dormant in any given season, they are essentially protected belowground and can easily withstand dry years, fires, herbivory, or other aboveground disturbances. Despite highly variable aboveground populations, belowground stages provide *Botrychium* populations with a high degree of buffering against local extinction. The belowground structure bank is a reserve of plants in various stages that can eventually produce an aboveground population, regardless of past aboveground perturbations.

Dyer (1994) noted the importance of fern propagule banks to the conservation of fern species. Reintroducing or augmenting populations from the spore bank broadens the options available for many species. Whereas it is difficult to manipulate *Botrychium* belowground structure banks, it is important to recognize the importance of these banks to the overall population dynamics. Modeled *Botrychium* populations (Johnson-Groh et al., 1998) predict greater stability of populations than would be concluded from monitoring only aboveground plants. This resiliency is a result of the large belowground reserve of gametophytes and juvenile sporophytes capable of regenerating the population. The long-term impact of environmental perturbations on populations is buffered by a large bank of belowground structures.

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Additions to the Fern Flora of Saba, Netherlands Antilles

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Abstract.—Recent fieldwork on Saba, Netherlands Antilles, has resulted in the discovery of nine fern species unrecorded for the island. All are known from other islands in the Antilles, as well as from other tropical areas.

Saba is a volcanic island about three kilometers in diameter that rises to about 850 m in a graceful cone called Mt. Scenery. The summit is the highest point in the Kingdom of The Netherlands. Saba lies south of Sint Maarten/St. Martin in the Windward Island chain at the northern end of the Lesser Antilles. About 1,300 people reside on Saba, which is little visited by tourists because of its small size and precipitous, rocky headlands that prevent beaches from forming. A beautiful fringing reef, recently declared a national park, makes the island a choice destination for SCUBA divers, but Saba also offers opportunities for hiking, birding, and botanising.

Although the island has been occupied for more than 350 years, a roller coaster-like road running the length of the island was hewed out of the rock and extensive retaining walls to contain the road were built by hand less than a half century ago. Prior to the road, all travel on the island was on foot, over trails that for the most part run rather circumferentially. Even so, the trails are by no means level because of the lava ridges and valleys between them, locally called guts. A day's hike usually includes elevation changes of at least 500 m. In recent years, additional trails have been constructed on the slopes and to the top of Mt. Scenery to make more of the island accessible to hikers. The road now connects the three principal villages with a dock and jetty at the southwestern end of the island, a minute beach (when it is exposed at all) on the western side, and a very short air field on the northeastern end, which is suitable only for short takeoff and landing aircraft. Ferry boats run from the dock and jetty.

Mt. Scenery is high enough to be covered by rain clouds for much of the time, and the island is therefore more moist than Sint Maarten/St. Martin and other nearby low islands that depend entirely upon convection for their rainfall. The additional water has resulted in a diverse native vegetation that has been divided into five types, which occur at different elevations above the sea as roughly concentric circles: grassy scrub near sea level, dry woodland above grassy scrub on the leeward side, moist forest above these types, rain forest above moist forest, and cloud forest at the summit of Mt. Scenery.

In the 19th and 20th centuries, much of the forest was destroyed by crop production or grazing, especially at lower elevations and where the upper slopes are not so steep. With improved access by boats, the residents of Saba imported more of their food, and cultivation and grazing ceased in the less accessible areas of the island. Native vegetation used to return slowly through a succession of relatively temporary associations of plants, the first of which was tree fern brakes (*Cyathea arborea* and *C. muricata*) (Hodge, 1990; Romeijn, 1989).

On the windward (eastern) side of the island, the forest vegetation was highly degraded by hurricanes in the 1990's. Many trees were uprooted, and those that remained were broken and battered. What was ferny, shaded forest floor is now almost fully open to the sun. Because of this, the dominant herbs are now Elephant Ear (*Philodendron giganteum*), various *Heliconia* species, and invasive vines and weeds. In the destroyed areas, there is little difference between the moist, rain, and cloud forests. All have open canopies, and weedy species are dominant on the ground. On the leeward (western) side of the island, the forests are much more intact, and it is still possible to distinguish between the forest types by their structure, moisture, dominant flowering plants, and complement of ferns.

Nephrolepis multiflora is often dominant in formerly cultivated fields on the windward slopes of Mt. Scenery, where it forms an impassable layer ca. 1 m tall that can persist for at least a decade and probably much longer. Only a few tree ferns appear to be capable of germinating and growing through the mat, but they form insufficient cover to make a tree-fern brake. They fail to shade and to kill the *Nephrolepis* and so cannot put in motion the normal succession to moist forest or rain forest.

The usual processes of succession at elevations below 600 m are also impeded by wild goats, which cause erosion and eat the leaves and shoots of young trees before they grow beyond reach, preventing regeneration of the forest (Romeijn, 1989). At present, hunting for meat is insufficient to overcome the goats' reproduction. A small part of the northern slopes of Mt. Scenery, encompassing all the vegetation types, and the entire summit of the mountain are now a protected park. Fencing this area to exclude goats would be good park management and would provide an opportunity for interesting ecological studies as well.

The principal botanical collectors who have visited Saba and collected pteridophytes are W. F. R. Suringar in 1885, I. Bolding in 1906, Bro. M. Arnoldo in 1946 and subsequent years, A. L. Stoffers in 1953, and G. R. Proctor in 1988 and 1996. Prior accounts of the pteridophytes of Saba were published by Kramer (1962) and by Proctor (1977). The latter author has also maintained an unpublished checklist of Saba plants, which he was very kind to share with me. The number of pteridophyte taxa known from Saba was 66, principally in the families Thelypteridaceae (12 taxa), Polypodiaceae (11 taxa), and Hymenophyllaceae (10 taxa).

At the suggestion of G. R. Proctor, my wife Jeannette and I spent several days in April, 1999 hiking trails and occasionally roadsides, mostly in the higher, forested parts of the island. (The grassy scrub might better be searched for ferns during the wet season in the winter.) The trails around the island are maintained by volunteers, and only the less travelled ones require the services of a guide. Considering how little botanising Saba has received, it is no surprise that we were able to find the species in the following list new to the island.

Ctenitis meridionalis (Poir. *in* Lam.) Ching.—Rare, terrestrial along trail between Sandy Cruz and Troy, near Down Gut above Wells Bay, in moist forest at 500–600 m elevation, *Lellinger 2034* (U, US).

Endemic in the Lesser Antilles.

Diplazium cristatum (Desr.) Alston.—Rare, terrestrial in shady places along trail above Upper Hell's Gate W and NW to beyond Sandy Cruz, in rain forest at 500–600 m elevation, *Lellinger 2033* (U, US).

Widespread in tropical America from northern South America northward.

Nephrolepis multiflora (Roxb.) Jarrett *ex* Morton.—Common along roadsides between Big Rendevous and English Quarter, in hurricane-damaged moist forest and rain forest at 400–600 m elevation, *Lellinger 2028* (U, US).

An Old World species naturalized sporadically in the Antilles, Mexico to Peru, and Brazil.

Pteris tripartita Swartz.—Seen along the trail in moist forest along the trail between Sandy Cruz and Troy.

An Old World species naturalized widely in the Antilles and from Nicaragua to Venezuela and Bolivia.

Thelypteris hispidula (Dcne.) Reed var. ***hispidula.***—Rare on street-side rock walls at The Level, SW of Windwardside, in shade at 400–500 m elevation, *Lellinger 2027* (U, US).

Widely distributed in tropical America.

Thelypteris poiteana (Bory) Proctor.—Occasional along the trail from Troy to Bottom Hill, terrestrial in moist forest at 450–500 m elevation, *Lellinger 2038* (U, US).

Widely distributed in tropical America.

Trichomanes krausii Hook. & Grev.—Rare along the trail between Sandy Cruz and Troy, near Down Gut above Wells Bay, on shady, clay banks in moist forest at 500–600 m elevation, *Lellinger 2037* (U, US).

Widely distributed in tropical America.

Trichomanes membranaceum L.—Rare along the trail between Sandy Cruz and Troy, near Down Gut above Wells Bay, on shady, clay banks in moist forest at 500–600 m elevation, *Lellinger 2035* (U, US).

Widely distributed in tropical America.

Trichomanes punctatum Poir *in* Lam. subsp. ***punctatum***.—Occasional along the trail between Sandy Cruz and Troy, near Down Gut above Wells Bay, on shady banks in moist forest at 500–600 m elevation, *Lellinger 2036* (U, US).

Distributed in the Antilles, Trinidad and Tobago, and Venezuela.

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Taxonomic Notes on Hawaiian Pteridophytes

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ABSTRACT.—A review of the literature regarding Hawaiian pteridophytes, examination of herbarium specimens, and much field work resulted in the need to publish two new varieties and to make thirteen new combinations.

In preparing a book reviewing the pteridophyte flora of Hawaii, the author examined type specimens, reviewed collections at several herbaria, consulted literature, observed common garden studies, and conducted much field work. Findings from these studies necessitate the publication of thirteen new combinations (*Adenophorus pinnatifidus* var. *rockii*, *Asplenium horridum* var. *glabratum*, *A. kaulfussii* f. *bipinnatum*, *A. kaulfussii* f. *gemmiparum*, *A. peruvianum* var. *insulare*, *Christella* ×*intermedia*, *Christella wailele*, *Dryopteris crinalis* var. *podosorus*, *D. glabra* var. *alboviridis*, *D. glabra* var. *hobdyana*, *Hypolepis hawaiiensis* var. *mauiensis*, *Microlepia strigosa* var. *mauiensis*, and *Microsorpum spectrum* var. *pentadactylum*) and the description of two new varieties (*Dryopteris glabra* var. *flynnii*, *and Polypodium pellucidum* var. *acuminatum*).

Adenophorus pinnatifidus Gaudich. var. **rockii** (Copel.) D. D. Palmer, *comb. nov.*

Polypodium rockii Copel. Philipp. J. Sci. 11:173. 1916. *Adenophorus* sarmentosus (Brack.) K. A. Wilson var. *rockii* (Copel.) O. Deg. & I. Deg., Fl. Hawaiiensis, fam. 17a. 1967.

Adenophorus pinnatifidus var. *rockii* is distinguished from var. *pinnatifidus* by its longer, narrower, more linear, acute-tipped segments arising at an oblique angle from the midrib, and often by the presence of more sori on the lobes (var. *pinnatifidus* with 3–4 sori per lobe, and var. *rockii* with 1–7).

Asplenium horridum Kaulf. var. **glabratum** (W. J. Rob.) D. D. Palmer, *comb. nov.*

Asplenium glabratum W.J. Rob., Bull. Torrey Bot. Club 40:214. 1913.

Asplenium glabratum is here recognized as a variety. It differs from *A. horridum* var. *horridum* in the degree of scaliness, var. *horridum* being very scaly and var. *glabrum* nearly glabrous. However, one can find a continuum of plants with intermediate degrees of scaliness between the two extremes. Hillebrand first recognized this taxon as *Asplenium horridum* Kaulf. var. β (Fl. Hawaiian Isl. 604. 1888).

Asplenium kaulfussii Schltdl.

This is an extremely variable species. Most plants are 1-pinnate with acute or obtuse-tipped pinnae, but some may produce pinnae with margins that are lacerate or deeply incised with toothed segments, and rarely the fronds may even be 2-pinnate. Usually nonproliferous, plants in some scattered colonies can produce a few proliferations or sometimes proliferations can be found on all pinnae. Variant fronds are sometimes found on the same rhizome as the typical 1-pinnate form. I have chosen to recognize these variants as forms of *A. kaulfussii*. The following key separates the forms.

1. Fronds 1-pinnate . . . . . 2.
1. Fronds 1-pinnate-pinnatisect to 2-pinnate . . . . . 3.
2(1). Pinna margins entire, lacking proliferations . . . . . f. kaulfussii
2. Pinna margins entire, proliferations occasional to plentiful on the lateral veins of pinnae . . . . . f. gemmiparum
3(1). Fronds fully 2-pinnate, the pinnules usually resembling small, typical pinnae . . . . . f. bipinnatum
3. Fronds 1-pinnate-pinnatisect to fully 2-pinnate, the ultimate segments linear with 2(4) obtuse lobes on an expanded apex . . . . . f. dareoides

Asplenium kaulfussii Schltdl. forma **bipinnatum** (Hillebr.) D. D. Palmer, *comb. nov.*

Asplenium bipinnatum Hillebr. Fl. Hawaiian Isl. 595. 1888. *A. lydgatei* Hillebr. Fl. Hawaiian Isl. 596. 1888. *A. meiotomum* Hillebr. Fl. Hawaiian Isl. 596. 1888.

This rare and variable form is fully 2-pinnate, with long-stalked pinnae and short-stalked pinnules that are smaller but of the same character as the pinnae of 1-pinnate fronds.

Hillebrand originally described *A. bipinnatum* and noted its relationship to *A. kaulfussii*. This isolated form of *A. kaulfussii* has ovate pinnae with 7 or 8 pinnules on each side. It was found in Niu Valley on Oahu, but has not been collected for many decades.

Asplenium kaulfussii Schltdl. forma **dareoides** (Hillebr.) W. H. Wagner, Contr. Univ. Michigan Herb. 22:160. 1999.

This rare form, not recently collected, is characterized by pinnatisect to 2-pinnate pinnae and linear ultimate segments with tips expanded into 2(4) obtuse lobes. Fronds of this form have on a few occasions been found on rhizomes producing the typical form.

Asplenium kaulfussii Schltdl. forma **gemmiparum** (Hillebr.) D. D. Palmer, *comb. nov.*

Asplenium kaulfussii Schltdl. var. *gemmiparum* Hillebr. Fl. Hawaiian Isl. 592. 1888. *A. enatum* Brack. U. S. Expl. Exped., Fil. 153 t. 21, f. 1. 1854.

A. enatum Brack. var. *gemmiparum* Hillebr. Fl. Hawaiian Isl. 593. 1888. *A. gemmiferum* Schrad. var. *enatum* (Brack.) O. Deg. & I. Deg., Fl. Hawaiiensis, fam. 17d. 1959. *A. mannii* Hillebr. var. *gemmiparum* Hillebr. Fl. Hawaiian Isl. 595. 1888.

This uncommon, localized, 1-pinnate form bears one or more proliferations on the adaxial surface on one or a few lateral veins of a single pinna, or on several pinnae including the apical one. There are forms with very few proliferations, but the variation in this character seems to be continuous, and nonproliferous fronds are sometimes found on the same rhizomes as proliferous ones.

Asplenium kaulfussii Schltdl. forma **kaulfussii**

This is the typical 1-pinnate form with entire pinnae, frond tips resembling lateral pinnae, and no proliferations. The pinnae tips vary from wide-obtuse, to long-acute.

Asplenium peruvianum Desv. var. **insulare** (C. V. Morton) D. D. Palmer, *comb. nov.*

Asplenium fragile C. Presl var. *insulare* C.V. Morton, Amer. Fern J. 37: 116. 1947. *A. fragile sensu* Hillebr., *non* C. Presl, Tent. Pterid. 108. 1836. *A. rhomboideum sensu auctt., non* Brack. U. S. Expl. Exped., Fil. 16:156, t. 21. 1854.

The name *Asplenium peruvianum* Desv. is recognized as having priority for the taxon commonly known as *A. fragile* C. Presl. Some authors have recognized *A. rhomboideum* Brack. as a synonym of *A. triphyllum* C. Presl, another fern in a group with close affinities *to A. peruvianum.*

Asplenium peruvianum var. *peruvianum* is native to the Peruvian Andes. The Hawaiian variety is larger and coarser, with thicker rachises, larger fronds, and a superior basal lobe on almost all the pinnae.

Asplenium peruvianum var. *insulare* may in fact include two taxa: a delicate, nonproliferous, longer, narrower, light green form often found in dark lava tubes, and a coarser, shorter, wider, darker green, usually proliferous form found in more open areas. This taxon is part of a group of closely related species that needs further study in Hawaii as well as in Peru.

Christella ×**intermedia** (W. H. Shieh & J. L. Tsai) D. D. Palmer, comb. nov.

Cyclosorus ×*intermedius* W. C. Shieh & J. L. Tsai, J. Sci. Engin. (Natl. Chung-Hsing Univ.) 24:8. 1987. *Christella* ×*incesta* (W. H. Wagner) Nakaike & Kawabata, J. Nat. Hist. Mus. Inst. Chiba 3(2):145. 1995. *Cyclosorus* ×*incestus* (W. H. Wagner) W. H. Wagner, Contr. Univ. Michigan Herb.

22:156. 1999. *Thelypteris* ×*incesta* W. H. Wagner, Contr. Univ. Michigan Herb. 19:79. 1993.

A book in preparation will include, and some older treatments would have included, this hybrid in the genus *Christella*, along with the related *C. cyatheoides*, *C. boydiae*, *C. dentata*, *C. parasitica*, and *C. wailele*.

This hybrid had been thought limited to Hawaii when the name *Thelypteris* ×*incesta* was published in 1993. However, the name *Cyclosorus* ×*intermedius*, identifying this hybrid in Taiwan and published in 1987 clearly has priority.

Christella wailele (Flynn) D. D. Palmer, *comb. nov.*

Thelypteris wailele Flynn, Contr. Univ. Michigan Herb. 20:246. 1995. *Cyclosorus wailele* (Flynn) W. H. Wagner, Contr. Univ. Michigan Herb. 22:156. 1999.

A book in preparation will include, and some older treatments would have included, this recently described fern in the genus *Christella*, along with the related *C. cyatheoides*, *C. boydiae*, *C. dentata*, and *C. parasitica*.

Dryopteris crinalis (Hook. & Arn.) C. Chr. var. **podosora** (W. H. Wagner) D. D. Palmer, *comb. nov.*

Dryopteris podosora W. H. Wagner, Contr. Univ. Michigan Herb. 20:252. 1995.

Dryopteris crinalis var. *podosora* may be distinguished from var. *crinalis* by sori that are globular and raised on conspicuous, narrow, pale, vascularized, stalks 0.8–1.3 mm long. All other characteristics of this taxon fall within the range of variation seen in var. *crinalis*.

Dryopteris crinalis var. *podosorus* is very rare and is restricted to wet, mossy walls near streams in damp, dark valleys near the Pihea Trail in the Kokee area of Kauai.

Dryopteris glabra (Brack.) Kuntze var. **alboviridis** (W. H. Wagner) D. D. Palmer, *comb. nov.*

Dryopteris alboviridis W. H. Wagner, Contr. Univ. Michigan Herb. 22:177. 1999.

Dryopteris glabra var. *alboviridis* differs from var. *glabra* by its light green color, coarser cutting with usually larger, wider ultimate segments (var. *alboviridis* 3–5 mm wide and var. *glabra* 2–3 mm wide) with crenate-dentate margins, and thicker and more leathery texture.

Careful study of this localized fern near the Pihea Trail in the Kokee area of Kauai has shown that while the polar form of the variety is distinct, a

continuum of forms blending into *D. glabra* var. *glabra* is found within a few hundred meters of plants typical of var. *alboviridis*.

Dryopteris glabra (Brack.) Kuntze var. **flynnii** D. D. Palmer, *var. nov.*—TYPE: Kauai: Waimea District: Kauaikinana Stream, W of Pihea Trail, *D. D. Palmer & J. Obata,* 14 Sept 1994, *K. Robinson 2139* (holotype BISH).

Dryopteris glabra var. *glabrae* similis sed differt lamina linearitriangulari atque plus elongata, stipite angustiore, segmentis ultimis angustioribus, et glandulis multo numerosioribus.

This variety is similar to *D. glabra* var. *glabra*, but differs in having linear-triangular and more elongated lamina, narrower stipes, narrower ultimate segments, and lamina covered with many more glands.

Plants delicate, drooping, very glandular, found on wet, dark stream banks. *Rhizomes* decumbent bearing sparse, brown, linear-triangular, 5–8 mm long scales. *Fronds* drooping, to 60 cm long. *Stipes* thin, 1–2 mm diam., stramineous to brown, glabrous, except for a few scattered scales at very base. *Blades* linear-triangular, 2–3 times as long as wide, 3–4-pinnate, pale green, very glandular; rachises thin, mostly 1–1.5 mm diam. *Pinnae* heavily clothed with globular, adnate glands, with multicellular, uniseriate hairs scattered on the veins; ultimate segments mostly less than 1.5 mm wide, except for wider anterior basal lobe, rounded to toothed at tips. *Sori* marginal to submarginal at tips of ultimate segments. *Indusia* glandular.

OTHER SPECIMENS EXAMINED.—**Hawaii:** Kauai: Waimea District: Kokee State Park, Kauaikinana Stream W of Pihea Trail, in *Metrosideros*-dominated wet forest with *Cheirodendron*, *Broussaisia*, *Cyanea*, *Melicope*, *Cibotium*, *Diplazium*, and *Athyrium*, elev. 1100 m, terrestrial on vertical, shaded, dripping walls along stream with *Athyrium microphyllum*, 23 July 1992, *T. Flynn & R. Hanna 5068, 5069, 5070* (PTBG). Pihea Trail between Pihea and the junction with the Alakai Swamp Trail, along small tributary of Kauaikinana Stream W of trail, in *Metrosideros*-dominated rain forest, elev. ca 1180 m, terrestrial on bank along stream in partial shade, 20 April 1990, *T. Flynn, D. Farrar, T. Ranker & D. Thomson 3866, 3866a* (PTBG). Kauaikinana Stream, at base of shaded, mossy wet wall, just above stream, 28 July 1992, *W. H. Wagner, F. S. Wagner, D. D. Palmer & T. Flynn 92117* (MICH).

Presently known only from a local population, growing between 1200–2000 m on the lower parts of shaded, mossy, wet banks along Kauaikinana Stream west of the Alakai Swamp Trail on Kauai.

The varietal name honors Tim Flynn, collection manager of the herbarium at the National Tropical Botanical Garden, who discovered this variety in 1992.

Fronds of this delicate and extremely glandular variety stick tightly to the papers they are dried in and must be removed with care. Its drooping habit, pale green color, fronds that are 2 to 3 times as long as wide, and its habitat on shady, wet banks distinguish it from the other varieties of *Dryopteris glabra*.

Dryopteris glabra var. **hobdyana** (W. H. Wagner) D. D. Palmer, *comb. nov.*

Dryopteris hobdyana W. H. Wagner, Contr. Univ. Michigan Herb. 22:177. 1999.

Study of this fern in its habitat on the upper slopes of Haleakala on Maui has shown that while the extreme manifestation of this variety is quite distinct, a continuum of plants with features blending into *Dryopteris glabra* var. *glabra* is found within a few hundred meters of plants typical of var. *hobdyana*.

A rhizome of this fern, removed from its habitat at an elevation about 2150 meters on the north slope of Haleakala—where it is exposed to trade winds, bright morning sun, afternoon cloudiness, and frequent freezing temperatures at night—was transplanted to a sheltered, less exposed, much warmer climate at Lyon Arboretum in upper Manoa Valley on Oahu. A few fronds typical of *D. hobdyana* were left attached to the rhizome. The very next frond that emerged from this rhizome, and several fronds since, were typical of var. *glabra*, suggesting that plants typical of *D. hobdyana* may be ecotype forms.

Further study is needed. For the present, this distinctive taxon is assigned the varietal name *D. glabra* var. *hobdyana*.

Hypolepis hawaiiensis Brownsey var. **mauiensis** (Hillebr.) D. D. Palmer, *comb. nov.*

Phegopteis punctata (Thunb.) Hillebr. var. *mauiensis* Hillebr. Fl. Hawaiian Isl. 663. 1888.

Hypolepis hawaiiensis var. *mauiensis* is a fully fertile miniature variety of *H. hawaiiensis*. Fertile fronds range from 6–25 cm long with blades that are 1-pinnate-pinnatifid to 3-pinnate. The rhizomes are slender (1–3 mm diam.) and long-creeping. The stipes, rachises, and costae are well covered with fine, tan, catenate (chain-like), acute-tipped hairs.

This rare fern occurs only on West Maui, where it was recently collected at an elevation of 1700 m on Puu Kukui in the Kapunakea Preserve; it was reported from Mount Eke by Hillebrand in 1888.

Microlepia strigosa (Thunb.) C. Presl var. **mauiensis** (W. H. Wagner) D. D. Palmer, *comb. nov.*

Microlepia mauiensis W. H. Wagner, Contr. Univ. Michigan Herb. 19:73, f. 6. 1993.

Microlepia mauiensis was described as a very hairy relative of *M. strigosa* with a somewhat zigzag rachis. Since its description, a continuum of intermediate forms from nearly completely glabrous to very hairy has been noted. Some plants have very hairy indusia and glabrous rachises, and the reverse is also seen. The degree of variation in hairiness suggests a variable species with a very hairy variety with a slightly zigzag rachis (*Microlepia strigosa* var. *mauiensis*) as one extreme manifestation.

Microsorum spectrum (Kaulf.) Copel. var. **pentadactylum** (Hillebr.) D. D. Palmer, *comb. nov.*

Polypodium spectrum Kaulf. var. *pentadactylum* Hillebr. Fl. Hawaiian Isl. 560. 1888.

The 5-lobed blades of var. *pentadactylum* distinguish it from var. *spectrum*, which has a triangular, 3-lobed blade. It is found in scattered areas on Kauai.

Polypodium pellucidum Kaulf. var. **acuminatum** D. D. Palmer, *var. nov.*—TYPE: Kauai: Waimea District, Koaie Stream in upper Waimea Canyon, 16 Apr 1991, *D. D. Palmer, B. Gustafson & staff of NTBG 586* (holotpe BISH).

Polypodium pellucidum var. *pellucidum* simulans sed differt lamina linearideltata, lobis acutis margine crenulatis, venis basalibus pinnarum basalium interdum anastomosantibus, et pagina abaxiali pilis brevibus vestita.

This variety resembles *P. pellucidum* var. *pellucidum*, but differs in having linear-deltate blades, acute lobes that are crenulate on the margin, basal veins of the basal pinnae sometimes anastomosing, and the abaxial surface clothed with short hairs.

Fronds to 55 cm long. *Rhizomes* long-creeping, 3–5 mm in diam., the scales light brown to tan, linear-lanceolate, narrowing to a hairlike tip. *Blades* linear-deltate, deeply pinnatisect to fully 1-pinnate at the base with 2–several lobes not winged to the rachises; *lobes* of blades up to 32, long, narrow, acuminate, with regularly crenulate to crenulate to serrate margins; false veins absent; fine, round-tipped hairs numerous on the abaxial surfaces of the lobes, costae, and veins, these 0.1 mm long, 3–7-celled, sparse on the adaxial surfaces; *veins* often anastomosing to form a few areolae at the bases of the lobes, especially lowest lobes; *sori* medial.

OTHER SPECIMENS EXAMINED.—**Kauai:** Waimea District: Koaie Canyon 1–2 miles above Lonomea Camp, elev. 520–580 m, in crevice of rock face above stream, 16 Apr 1991, *T. Flynn, D. Lorence, S. Perlman, K. Wood, D. Palmer & B. Gustafson 4532* (NTBG). **Maui:** Haleakala, Kaupo Gap near National Park boundary, adjacent to the trail, elev. ca. 1525 m, 3 Jun 2000, *D. D. Palmer with H. Oppenheimer, T. Ranker, J. Ott, & P. Welton 3162* (Hb. Palmer).

*Polypodium pellucid*um var. *acuminatum* differs from var. *pellucidum* by having fully 1-pinnate fronds with 2 to several lobes not winged to the rachises; long, narrow, pointed lobes; veins sometimes anastomosing at the bases of some lower lobes; the lack of "false veins"; and the presence of numerous, minute, 0.1 mm long, 3-celled, round-tipped hairs common over the abaxial surfaces of the lobes, costae and veins.

Although Hillebrand's *Polyodium pellucidum* var. γ (Fl. Hawaiian Isl. 558. 1888) is not a legitimate name, he was the first to recognize this variety.

This little-known variety is not well studied and is known only from isolated areas, 500–580 m, in the Waimea, Kokee, and Hanalei districts

of Kauai, and the Kaupo Gap area of Haleakala on East Maui at 1525 m elevation.

Plants with characteristics between var. *pellucidum* and var. *acuminatum* are found. The minute hairs characteristic of this variety are found in occasional scattered populations of the usually glabrous var. *pellucidum*. In these populations the hairs may be scattered or numerous.

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Novelties in Pteridaceae from South America

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ABSTRACT.—Three new species (*Adiantum squamulosum*, *Pteris boliviensis*, and *P. krameri*) from South America are described and illustrated. We also provide a new name for an endemic species of *Cheilanthes* from Brazil and a new record of *Pteris* for Bolivia.
RESUMO.—Três novas espécies (*Adiantum squamulosum*, *Pteris boliviensis* e *P. krameri*) da América do Sul são descritas e ilustradas. São também apresentados um nome novo para uma espécie endêmica de *Cheilanthes* do Brasil e um novo registro de ocorrência para *Pteris* na Bolívia.

Continuing studies with South American Pteridaceae, especially from Bolivia and Brazil, indicate that the following taxa are either undescribed, need a new name, or constitute a new country record. We hereby record these additions and dedicate this paper to the memory of Warren (Herb) Wagner Jr., who contributed to our understanding of *Pteris*, especially in Hawaii and Florida.

Adiantum squamulosum Prado & A. R. Sm., *sp. nov.* (**Fig. 1**).—TYPE: BOLIVIA: Depto. Beni: Pcia. Vaca Diez: 13 km E of Riberalta on road to Guayaramerín, then 3 km N on side road, 10°58′S, 66°58′W, 230 m, 27 May 1982, *J. C. Solomon 7818* (holotype MO on 2 sheets; isotypes LPB not seen, UC).

Ex affinitate *A. diogoano* Glaz. ex Baker, stipitibus et rachibus dense rufosquamulosis, pinnulis abaxialiter et adaxialiter rufosquamulosis, squamis valde ciliatis aut setosis, pinnulis 41–46-jugatis (vs. 21–24-jugatis), indusiis squamulosis (vs. pilosis) differt.

Plants terrestrial. Rhizomes short-creeping, ca. 0.6 mm in diam., scaly, the scales somewhat shiny, essentially concolorous, appressed throughout or spreading distally, varying from medium to dark brown, lanceate, sparsely denticulate at margins. Fronds monomorphic, 2-pinnate, 73–116 cm long, the laminae 25–35 cm wide; stipes approximate, 1/2–2/3 the length of the frond, black, adaxially sulcate, densely scaly, the scales appressed throughout, concolorous, ferrugineous, 3–4 mm long, linear to narrowly lanceate with a filiform apex, ciliate at margins and with several processes at the base; rachises similar to stipes and their indument similar; pinnae oblong-lanceate, slightly narrowed at their base, tapering at the apex, (8)15–30 × 1.8–3.2 cm, the lateral pinnae (3)4–9 pairs, ascending, alternate, the terminal

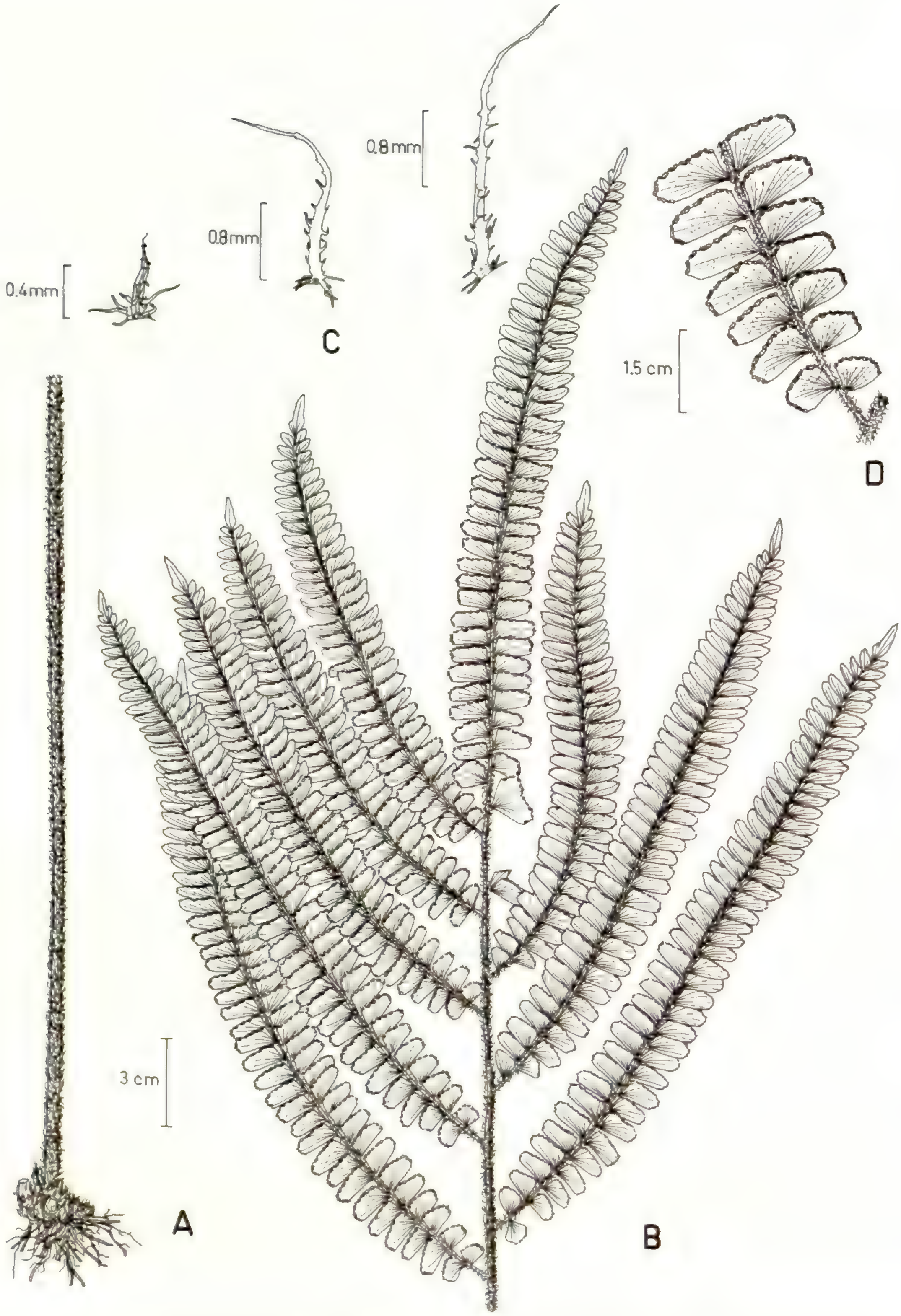


FIG. 1. *Adiantum squamulosum*. A. Rhizome and part of stipe; B. Distal part of fertile frond; C. Rachis scales; D. Abaxial view of fertile pinnules.

pinna conform, 1–1.5 times longer than the subtending pinnae, 0.8–1 times as long as medial pinnae; indument of costae like that of stipes and rachises; pinnules (11)41–46 pairs per pinna, ca. 2 times longer than wide, chartaceous, not articulate, free-veined, without an evident midrib, the proximal pinnules reduced, somewhat rounded or rhombic, the medial pinnules dimidiate, trapeziform to oblong and with the acroscopic base truncate, the sterile apices obtuse to acute, sterile margins denticulate, fertile apices angular, distal pinnules ca. 1/2 or less as long as the medial pinnules; both pinnule surfaces scaly, the scales sparse, ferrugineous, 1.0–1.5 mm long but otherwise similar to those of the stipes, glands absent, veins slightly prominulous, idioblasts present between the veins; sori oblong, up to 7(9) per pinnule; indusia scaly, scales with filiform apices and pectinate bases, entire to erose, the cells well evident; spores yellowish, trilete, tetrahedral-globose, with prolonged angles, the surface rugulate.

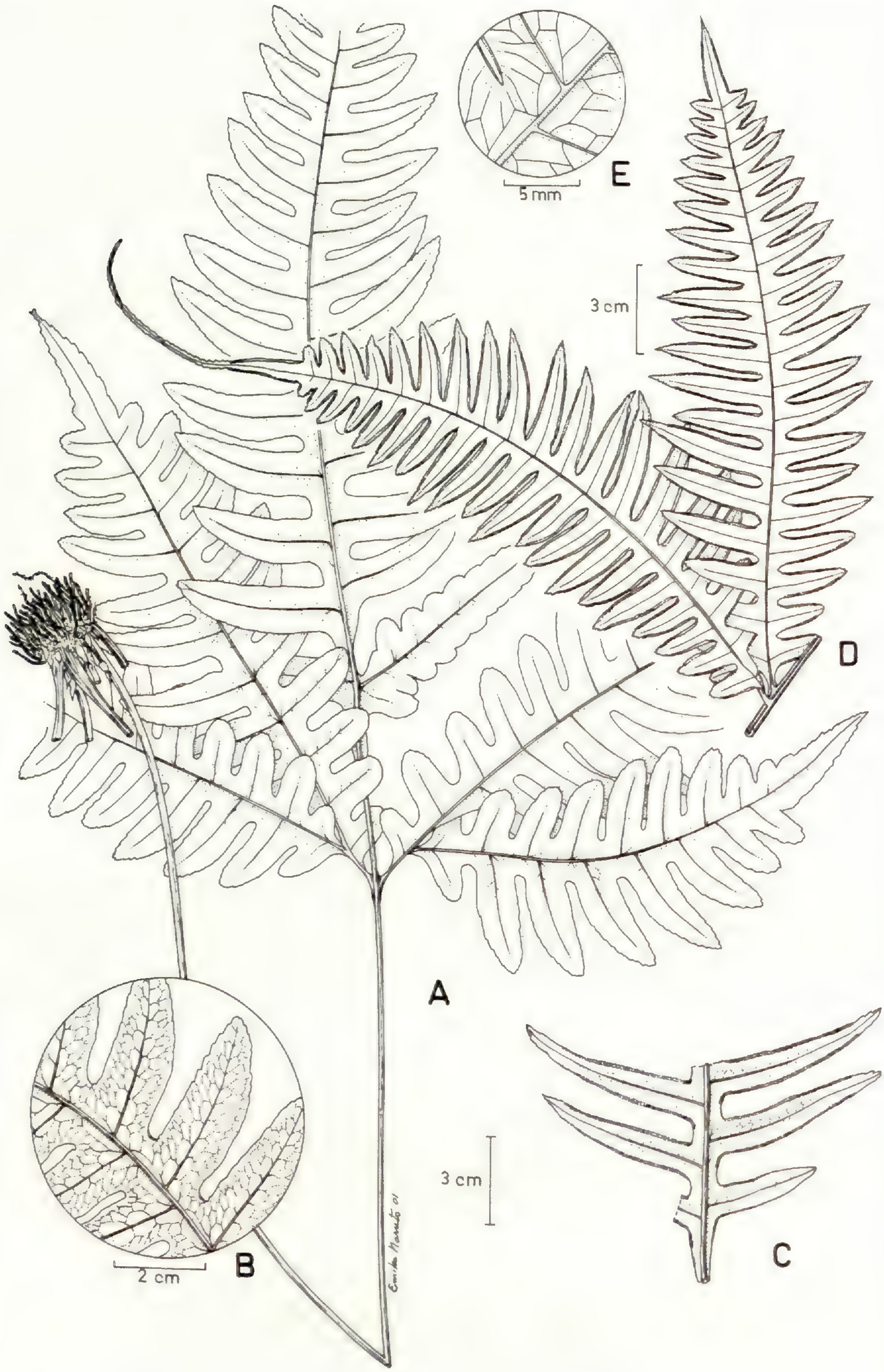
Adiantum squamulosum is distinguished by having densely scaly stipes and rachises, large scales on both surfaces of the pinnules, a relatively large number of pinnule pairs per pinna (up to 46), and scaly indusia. This species occurs in partially disturbed, primary, non-inundated forest at low elevations (ca. 230 m) along road margins. *Adiantum diogoanum* Glaz. *ex* Baker is probably the most closely related species but differs in having stipes, rachises, and pinnules glabrescent, fewer pinnule pairs per pinna (up to 24), and indusia with reddish hairs, rather than scales. It grows in drier, inland forests along rivers and slopes.

Adiantum squamulosum is known from a single locality in Bolivia, whereas *A. diogoanum* has a wide range in Brazil, occurring in the states of Pernambuco, Alagoas, Minas Gerais, São Paulo, and Paraná. The type was cited by Smith et al. (1999, p.247) as questionably *A. humile* Kunze.

Pteris boliviensis Prado & A. R. Sm., *sp. nov.* (**Fig. 2A–C**).—TYPE: BOLIVIA: Depto. Cochabamba: Prov. Chapare, 22 Feb 1929, 1700 m, *J. Steinbach 9327* (holotype UC).

Ad *P. lividam* Mett. affinis, a qua imprimis frondibus pinnatis (vs. tripartitis) et 2 vel 3 areolae inter contiguas costulas (vs. 1 areola) differt.

Plants terrestrial. Rhizomes short-creeping, densely clothed at apex with light brown ovate-lanceate scales 2–3 mm long, the scale margins entire and glabrous. Fronds erect, to ca. 52 cm long; stipes ca. 1/2 of frond length, up to 3 mm in diam., straw-colored, dark brown at base, grooved adaxially, smooth, glabrous except for a few ovate-lanceate scales at base; laminae chartaceous, 2-pinnate-pinnatifid at base, with 1–4 pinna pairs per frond, 1-pinnate-pinnatifid above the base, ending in a broadly based, pinnatifid apical pinna; rachises similar to stipes, alate distally, glabrous; proximal pinna pair stalked (stalks ca. 0.5 cm long), opposite, deeply pinnatifid, furcate, the basal basiscopic pinnules 12–16 × 4.5–7.0 cm, the remainder of the pinnae 20–22 × 5–7 cm; median pinnae sessile, subopposite, deeply pinnatifid, decurrent on the rachis, 13–14 × 5.5–6.5 cm; distal pinnae sessile, alternate,


5 mm
E
3 cm
D
A
2 cm
B
3 cm
C

decurrent on the rachis, 8–9 × 2.5–3.0 cm; costae not sulcate on both sides, glabrous, awns absent adaxially; pinna segments subopposite to alternate, oblong, the margins entire at the middle to crenate-serrate at the apex, the terminal segment of each pinna narrowly deltate, acute or acuminate, sinuses between segments roundish to biangulate; venation copiously areolate but free near margins and segment apices, with 2 or 3 costal areoles between adjacent costules, the costules slightly prominent especially on abaxial surfaces. Sori interrupted at sinuses and absent at apex of segments; indusia greenish when young, margins entire; spores brown to tan, trilete, tetrahedral, the surfaces rugulate with roundish tubercles and a smooth equatorial flange.

PARATYPE.—BOLIVIA. **Cochabamba:** Pcia. José Carrasco Torrico: 137 km antigua carretera Cochabamba-Villa Tunari, 17°06′S, 65°35′W, 1600 m, 18 July 1996, *M. Kessler et al. 7393* (paratype UC; isoparatype LPB not seen).

The two or three areoles between adjacent costules distinguish this species from *Pteris livida* Mett., which has only one areole between adjacent costules. In addition, the fronds are ternate in *P. livida* and pinnate in *P. boliviensis*.

Pteris boliviensis grows in wet forests and cleared forests at ca. 1600–1700 m.

Pteris krameri Prado & A. R. Sm., *sp. nov.* (**Fig. 2D, E**).—TYPE: GUYANA: Upper Essequibo Region: Rewa River, near camp 2 at foot of Spider Mountain, forest on light brown sand, 03°08′N, 0°58′W, 16 Sept 1999, *M. J. Jansen-Jacobs et al. 5923* (holotype UC; isotype U on 2 sheets).

A *P. altissima* Poir. costis aristis adaxialiter carentibus differt.

Plants terrestrial. Rhizomes stout, woody, creeping, densely clothed at apex with linear-attenuate, bicolorous scales, 3–5 mm long, these with a blackish to reddish brown central portion and entire to more or less erose, glabrous, pale margins. Fronds erect, ca. 1.3 m long; stipes ca. 2/3 of frond length, up to 6 mm in diam., straw-colored, grooved adaxially, smooth, glabrous except for a few bicolorous scales at base; laminae chartaceous, 2-pinnate-pinnatifid at base, 3–7 pinna pairs per frond, the median portion 1-pinnate-pinnatifid, fronds ending in a broadly based pinnatifid apical pinna; rachises similar to stipes, narrowly alate distally, glabrous or with sparse, whitish hairs ca. 1 mm long; proximal pinna pair stalked (stalks ca. 1.0 cm long), opposite, deeply pinnatifid, furcate, the basiscopic pinnules 12–22 × 3.7–6.0 cm, the remainder of the pinnae 29–35 × 7.5–10.0 cm; median pinnae sessile, subopposite, deeply pinnatifid, 22–30 × 7.5–8.5 cm; distal pinnae

←

FIG. 2. *Pteris* species. A–C. *Pteris boliviensis*. A. Habit; B. Detail of a sterile pinnae showing veins; C. Detail of fertile segments. D–E. *Pteris krameri*. D. Median pinnae; E. Adaxial view of lamina showing veins, costa, and costules.

sessile, alternate, 13–14 × 3.9–4.5 cm; costae not sulcate adaxially, glabrous or with sparse, minute, whitish hairs ca. 1 mm long, awns absent; segments subopposite to alternate, long-lanceate, the margins entire to conspicuously serrate at apex in fertile fronds (sterile fronds not seen), the terminal segment of each pinna elongate-deltate, acute to long-acuminate or sometimes caudate, the sinuses between the segments acute to roundish; venation copiously areolate but free near margins and segment apices, with 2 or 3 areoles between adjacent costules, veins slightly prominent especially on abaxial surfaces. Sori interrupted at sinuses and absent at segment apices; indusia greenish or brownish, the margin entire; spores tan, trilete, tetrahedral, the surfaces rugulate with roundish tubercles and smooth equatorial flange.

Pteris krameri is apparently most similar morphologically to *P. altissima* Poir., and both species occur in similar habitats: near river and stream margins, moist ravines, in rocky soils or silt from big rocks, or on humus-rich soils in forests. However, *P. altissima* differs in having an awn at the base of each costule on the adaxial side of the lamina. Although it has been inadequately assessed, the character of presence or absence of awns along the penultimate axes seems of fundamental importance in assessing relationships in *Pteris*, so the apparent similarity of *P. krameri* to *P. altissima* may be the result of convergent evolution. Other New World species that lack awns adaxially, and that have pinnatifid pinnae or pinnules, are *P. angustata* (Fée) C. V. Morton, *P. boliviensis* Prado & A. R. Sm., *P. brasiliensis* Raddi, *P. congesta* Prado, *P. decurrens* C. Presl, *P. denticulata* Sw., *P. fraseri* Mett. ex Kuhn, *P. lechleri Mett., P. leptophylla* Sw., *P. limae* Brade, and *P. pearcei* Baker, all species of South America and especially Brazil (Prado and Windisch, 2000). None of these species seems closely related or similar morphologically to *P. krameri*. Whether the presence or absence of adaxial awns on the lamina is a character that circumscribes monophyletic groups is an intriguing but unanswered question.

Although *Pteris krameri* is known only from the type gathering, it may have a wider range in northern South America. The species epithet honors the late Karl Kramer, who contributed much to the study of fern systematics and made *Pteris* one of his specialties; Dr. Kramer also focused especially on the ferns of the Guianas, the source of this new species.

Cheilanthes bradei Prado & A. R. Sm., *nom. nov.* for *Cheilanthes venusta* (Brade) R. M. Tryon & A. F. Tryon, *nom. illeg.*, which is based on *Notholaena venusta* Brade, Anais Reunião Sul-Amer. Bot. 2: 7, t. 4, f. 1,2. 1940. *Cheilanthes venusta* (Brade) R. M. Tryon & A. F. Tryon, Rhodora 83: 133. 1981, non Fée, Cat. Foug. Mex (Mém. Foug. 9): 12. 1857.—TYPE: BRAZIL: Estado de Minas Gerais: Diamantina, ad rupibus, 1400 m, Jun 1934, *A. C. Brade 13494* (holotype RB, not seen, frag. HB!).

This rather uncommon species can be distinguished by its delicate, terete stipes with uniseriate, jointed hairs, laminae proximally with 1–3 pairs of free pinnae and distally pinnatifid. It is endemic to southeastern Brazil

(Minas Gerais). The new name proposed below honors Alexander Curt Brade, who described this species in *Notholaena* (Brade, 1940).

SPECIMENS STUDIED.—BRAZIL. **Est. Minas Gerais**: Estrada Diamantina–Curvelo, *Williams & Anderson 8466* (UB); Santana do Riacho, Serra do Cipó, Parque Nacional da Serra do Cipó, Cachoeira da Farofa, CFSC 10240, *Prado et al.* (SPF); *Idem,* Serra do Cipó, *Sena s.n.* (RB, HB); *Idem, Schwacke 14520* (BHCB); Ouro Preto, Itacolomi, *Schwacke 9906* (RB); Lima Duarte, *Krieger s.n.* (BHCB); Mun. Gouveia: Rib. do Tigre, *Hatschbach 27841* (MBM, UC).

Pteris bakeri C. Chr.

According to Tryon and Stolze (1989. p. 80), *Pteris bakeri* C. Chr. is endemic to Peru and occurs in forests at middle to high elevations, 2300–3000 m. This is the second collection outside of Peru; Arbeláez (1996) also reported this species from the Colombian Andes.

Pteris bakeri can be distinguished by fronds ca. 1 m long, laminae decompound (5-pinnate at the base), very small ultimate segments 1–1.5 mm wide, free veins, and spiculate, scaly axes abaxially.

SPECIMENS STUDIED.—BOLIVIA. **Depto. Cochabamba**: Pcia. José Carrasco Torrico: 116 km antigua carretera Cochabamba–Villa Tunari, 17°08′S, 65°38′W, 2350 m, *Kessler et al. 7064* (LPB not seen, UC); *Idem*, 123 km antigua carretera Cochabamba–Villa Tunari, 17°08′S, 65°37′W, 2100 m, *Kessler et al. 7117* (LPB not seen, UC).

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Is Gametophyte Sexuality in the Laboratory a Good Predictor of Sexuality in Nature?

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ABSTRACT.—A previous study examined the sexual expression of cultured gametophytes of the Hawaiian endemics *Sadleria cyatheoides* and *S. pallida* grown on mineral-enriched agar. In the present work, we conducted field studies on the sexual expression of natural populations of *Sadleria* spp. gametophytes. Our primary goal was to compare field-collected data to the laboratory-based data of the earlier study to assess if the laboratory data were an accurate reflection of what is occurring in nature. Our results suggested that for generally inferring mating systems operating in nature, agar-based laboratory studies of gametophytes lead to the same conclusions as would observations of field-collected gametophytes. For detailed studies of gametophyte sexuality and development, however, an agar-based medium produces significantly different results than what is found among natural populations, although this may be true for any lab-based study regardless of growth medium. Thus, we suggest caution in the use of agar as a growth medium, and the use of laboratory conditions in general, for studies of fern gametophyte sexual development.

The gametophytes of homosporous ferns possess the ability to be either unisexual (antheridiate or archegoniate) or bisexual. In addition, they may undergo developmental sequences involving changes in sexuality over time due to genetic and/or environmental factors (Klekowski, 1969a; Greer & McCarthy, 1999). Because gametophytes of most species are easy to grow in culture, many studies have been conducted on cultured gametophytes to assess such factors as sexual expression/ontogeny (e.g., Klekowski, 1969b), antheridiogen production and response (e.g., Stevens & Werth, 1999), and populational genetic load/isolate potential (Peck et al., 1990). Numerous studies have employed data from lab-cultured gametophytes to make inferences about mating systems operating in nature (e.g., Soltis & Soltis, 1990, and references therein; Chiou et al., 1998; Li & Haufler, 1999). Although many studies have examined gametophytes that were cultured on mineral-enriched agar, several have employed various natural substrates (Rubin & Paolillo, 1983; Greer, 1993; Pangua et al., 1994; Lindsay & Dyer, 1996; Greer & McCarthy, 1999; see also Dyer, 1979, and references therein). A few studies have combined genetic data from natural populations of sporophytes with laboratory data from cultured gametophytes to assess the relationship between gametophytic attributes and levels and patterns of populational genetic variation (e.g., Haufler & Soltis, 1984, Ranker et al., 1996). Nearly all studies that employ data from lab-cultured gametophytes have at least one assumption in common: that the patterns and processes observed in the laboratory are indicative of what is occurring in nature. At least one study, however, has demonstrated significant differences in the sexual expression of gametophytes

collected from nature versus those cultured on mineral-enriched agar. Schneller (1979) found that 79.9% of gametophytes of *Athyrium filix-femina* (Woodsiaceae) collected from the wild were sexual, whereas those cultured were only 60.1% sexual. The proportions of gametophytes that were asexual, antheridiate, archegoniate, or bisexual differed between the two populations: wild-collected were 21.1% asexual, 49.6% antheridiate, 20.6% archegoniate, and 8.6% bisexual; lab-cultured were 39.9%, 30.5%, 27.3%, and 2.3%, respectively. These two distributions differ statistically (2×4 contingency table, lab vs. field by sexual category, $\chi^2 = 100.55$, df = 3, $p \ll 0.001$; our analyses). That study demonstrates that, at least for gametophyte populations of *A. filix-femina*, the sexual expression of gametophytes cultured on mineral-enriched agar was not a good indicator of the sexual expression of gametophytes occurring in the wild population studied. Such comparisons are crucial to assess the veracity of data collected from artificially cultured gametophytes for inferring patterns and processes occurring in nature.

Ranker et al. (1996) studied the sexual expression of lab-cultured gametophytes of two species of the endemic Hawaiian genus *Sadleria*, *S. cyatheoides* Kaulf. and *S. pallida* Hook. & Arn., grown on mineral-enriched agar. That substrate was chosen primarily for the sake of convenience and the ease of gametophyte manipulation. Observations were made on a total of 5,749 gametophytes from 26 sibships of *S. cyatheoides* and 3,032 gametophytes from 15 sibships of *S. pallida* (see Ranker et al., 1996, for explicit methods). All gametophytes were sampled from multigametophyte cultures. Although the two species differed statistically in the exact proportions of gametophytes that were antheridiate, archegoniate, or bisexual, the overall pattern among sexual gametophytes was the same in the two species: a small proportion was antheridiate (grand means of 9.7% and 10.0% from *S. cyatheoides* and *S. pallida*, respectively), a larger proportion was archegoniate (84.7% and 81.2%), and a small proportion was bisexual (5.6% and 8.8%). The predominance of unisexual gametophytes was consistent with analyses of sporophyte-population surveys of allozymic variation from which Ranker et al. (1996) inferred that sporophytes of both species primarily arose via intergametophytic matings.

Another aspect of the study of Ranker et al. (1996) involved assessments of the ability of *Sadleria* spp. gametophytes to produce and respond to antheridiogen (Döpp, 1950; Näf, 1979). Based on an excess of antheridiate gametophytes in treatment vs. control cultures, Ranker et al. (1996) concluded that an antheridiogen system was operating in these species. These results are at least superficially incongruent with the data from the sexual expression studies described above; that is, one might expect to find a greater proportion of antheridiate gametophytes from multi-gametophyte cultures, due to the action of antheridiogen, than was observed in either species.

We conducted field studies on the sexual expression of natural populations of *Sadleria* spp. gametophytes. Our primary goal was to compare field-collected data to the lab-based data of Ranker et al. (1996) to determine if the laboratory data were an accurate reflection of what is occurring in nature.

In particular, we were interested in comparing gametophyte populations cultured in the laboratory to those sampled in the field for: 1) the ratio of asexual to sexual gametophytes; 2) the ratio of unisexual to bisexual gametophytes, among sexual gametophytes and, 3) the ratio of antheridiate to archegoniate gametophytes, among unisexual gametophytes.

Materials and Methods

Five populations of *Sadleria* spp. sporophytes were located on the Hawaiian Island of Kauai in November, 1999. Three populations were mixtures of *S. cyatheoides* and *S. pallida* (populations CP-1, CP-2, and CP-3), one population was pure *S. squarrosa* (population S-1), and one population was pure *S. unisora* (population U-1). Populations CP-1, CP-2, CP-3, and U-1 were from the Hanalei District and population S-1 was from the Waimea District; exact localities are available from the first author. Samples of earthen or bryophyte-covered substrate approximately 10 cm by 10 cm by 1–2 cm (deep) were collected with a kitchen spatula from the vicinity of *Sadleria* sporophytes. Five to 10 of such samples were collected at each site. Samples were transported in plastic bags to the herbarium of the National Tropical Botanical Garden. Each sample was then inspected under a binocular dissecting microscope for the presence of *Sadleria* gametophytes, which could be distinguished from those of other species of ferns by the presence of distinctive glandular trichomes that are present on both gametophytes and sporophytes. *Sadleria* gametophytes were gently removed from the substrate, rinsed in water, and categorized under a compound microscope as belonging to one of four classes of sexuality: asexual, antheridiate, archegoniate, or bisexual. Only gametophytes with mature archegonia and antheridia were scored as bisexual.

Results

We found and recorded observations from a total of 200 gametophytes across the five field populations surveyed, with individual sample sizes ranging from 26 to 67 (Table 1). All gametophytes sampled possessed notch meristems.

Asexual vs. Sexual Gametophytes.—The percent ratio of asexual to sexual gametophytes in individual field populations ranged from 4:96 to 41:59 and, summed across populations, that ratio was 20:80 (Table 1). The results summed across populations were significantly different from what was observed in the laboratory, where nearly the opposite percent ratio (74:26, asexual:sexual) was obtained across all sibships of both species (2×2 contingency table of asexuality/sexuality vs. lab/field, $\chi^2 = 286.6$, df = 1, $p \ll 0.001$). Similarly, the results summed across just those field populations likely to be mixtures of gametophytes that were conspecific with those cultured (CP populations) also exhibited a percent asexual:sexual ratio (18:82)

TABLE 1. Sexuality of sampled gametophytes.

Population	N[1]	Asexual[2]	Sexual[2]	Antheridiate[3]	Archegoniate[4]	Bisexual[5]
Field results:						
CP-1	49	10 (20)	39 (80)	34 (87)	1 (3)	4 (10)
CP-2	26	1 (4)	25 (97)	9 (36)	13 (52)	3 (12)
CP-3	67	14 (21)	53 (79)	41 (77)	9 (17)	3 (6)
S-1	27	11 (41)	16 (59)	15 (94)	1 (6)	0
U-1	31	3 (10)	28 (90)	27 (96)	0	1 (4)
Total	200	39 (20)	161 (80)	126 (78)	24 (15)	11 (7)
Laboratory results[4]:						
S. cyatheoides	5749	4375 (76)	1374 (24)	133 (10)	1164 (85)	77 (5)
S. pallida	3032	2089 (69)	943 (31)	94 (10)	766 (81)	83 (9)
Total	8781	6464 (74)	2317 (26)	227 (10)	1930 (83)	160 (7)

[1] Sample size.
[2] Number (percent of total rounded to nearest whole percent).
[3] Number (percent of sexual gametophytes rounded to nearest whole percent).
[4] Data from Ranker et al. (1996), summed across sibships.

that was significantly different from that of laboratory gametophytes (χ^2 = 220.9, df = 1, $p \ll 0.001$). The percent ratio of asexual:sexual summed across laboratory sibships of *S. cyatheoides* was 76:24 and that in *S. pallida* was 69:31.

UNISEXUAL VS. BISEXUAL GAMETOPHYTES.—Among the sexual gametophytes sampled, all field populations exhibited a predominance of unisexual gametophytes (Table 1). Field populations did not differ statistically from laboratory populations in the ratio of unisexual to bisexual gametophytes, both summed across all field populations (χ^2 = 0.001, $p > 0.05$) and summed across just the CP populations (χ^2 = 0.462, $p > 0.05$).

ANTHERIDIATE VS. ARCHEGONIATE GAMETOPHYTES.—As with the ratios of asexual to sexual gametophytes, field-collected and lab-cultured gametophytes exhibited nearly opposite percent ratios of antheridiate to archegoniate gametophytes. The overall percent ratio in the field was 84% antheridiate to 16% archegoniate, among CP populations that ratio was 79:21, and that in the laboratory was 11:89. These field and laboratory proportions were significantly different from each other in total (χ^2 = 584.2, df = 1, $p \ll 0.001$) and when comparing only CP populations to laboratory populations (χ^2 = 397.6, df = 1, $p \ll 0.001$).

DISCUSSION

The results of our study of field-collected populations of gametophytes have important implications for the use and interpretation of data from lab-cultured gametophytes of *Sadleria* spp. and, possibly, of other taxa as well.

ASEXUAL VS. SEXUAL GAMETOPHYTES.—The striking difference between laboratory and field gametophytes in the relative proportions of asexual-to-sexual

gametophytes suggests that the laboratory conditions employed by Ranker et al. (1996) were a poor mimic of the natural environment in terms of simply allowing or forcing gametophytes to become sexual. Those laboratory conditions were similar to those that have been employed by numerous other investigators in studies of fern gametophytes. Thus, some factor or combination of factors in our laboratory cultures appears to have been inhibiting the development of sexual organs. One potential impact on inferences resulting from the laboratory data is that they would lead to an underestimate of the sexual reproductive potential of a population or species relative to those based on field observations, although Ranker et al. (1996) did not explicitly make any such inferences.

UNISEXUAL VS. BISEXUAL GAMETOPHYTES.—In both laboratory and field populations of gametophytes there was a predominance of unisexual gametophytes among those that were sexual. These results are similar to those from *A. filix-femina* (Schneller, 1979) and from *Blechnum spicant* (Blechnaceae; Cousens, 1979, 1981). Among sexual field-collected gametophytes of *B. spicant*, 21% were bisexual and 79% were unisexual (Cousens, 1981). Cousens (1979) agar-cultured gametophytes of *B. spicant* as isolated gametophytes and at moderate and high densities. Among sexual isolates, only 8% were bisexual and among both groups of multi-gametophyte cultures, 20% exhibited bisexuality. In terms of assessing the likelihood of unisexuality vs. bisexuality and, thus, the relative likelihood of intergametophytic mating vs. selfing, gametophytes cultured on mineral-enriched agar appear to behave in a manner consistent with development of gametophytes in nature.

ANTHERIDIATE VS. ARCHEGONIATE GAMETOPHYTES.—Among gametophytes of *Sadleria* spp. and *A. filix-femina* (Schneller, 1979), there were significant differences in the ratio of antheridiate-to-archegoniate gametophytes in the laboratory vs. in the field [using Schneller's data from *A. filix-femina*, we analyzed a 2 × 2 contingency table of antheridiate vs. archegoniate by lab vs. field; $\chi^2 = 29.07$, df = 1, $p < 0.001$]. In both cases, laboratory populations were generally mostly archegoniate and field populations were mostly antheridiate. These results are similar to those of Rubin and Paolillo (1983) where unisexual gametophytes of *Onoclea sensibilis* (Woodsiaceae) that were agar-grown were disproportionately archegoniate whereas those that were soil-grown were disproportionately antheridiate. Also, in field populations of *B. spicant* unisexual gametophytes were predominantly antheridiate (Cousens, 1981). The relative proportions of antheridiate vs. archegoniate gametophytes in laboratory populations of *B. spicant*, however, varied with density (Cousens, 1979). Among isolated gametophytes, all were archegoniate and all but one (98%) were archegoniate when cultured in moderate density (only one was antheridiate). By contrast, when cultured at high density, 96% of unisexual gametophytes were antheridiate and only 4% were archegoniate, similar to gametophytes collected from nature. Cousens attributed these differential patterns to a greater likelihood of antheridiogen effects at higher densities in the laboratory.

Laboratory studies of *S. pallida*, *S. cyatheoides* (Ranker et al., 1996), *A. filix-femina* (Schneller, 1979), and *B. spicant* (Cousens, 1979) have demonstrated that gametophytes of all of these species can produce and respond to the male-inducing pheromone antheridiogen, as has been shown for many other species of ferns (e.g., Näf, 1979; Schneller et al., 1990). Thus, as was suggested for *B. spicant*, it is possible that antheridiogen has a greater effect in natural populations of *Sadleria* spp. and *A. filix-femina* than on agar-cultured populations. The possibility that an agar-based medium directly inhibits antheridia formation and/or promotes archegonia formation, however, cannot be ruled out (see Rubin & Paolillo, 1983).

Similarly, the impact of potential differences in the age structure of natural and cultured populations on sexual expression should also be considered. In laboratory cultures, gametophyte populations typically are established from single sowings of spores, resulting in nearly even-aged populations. In nature, gametophyte populations would presumably be composed of mixed-age individuals. Because gametophytes do not respond to antheridiogen after they reach the notch-meristem stage and because most gametophytes in an even-aged stand would reach that stage essentially simultaneously, few cultured gametophytes would become antheridiate due to antheridiogen response. That would be particularly true at low to moderate densities. By contrast, in a mixed-aged natural stand, antheridiogen-producing gametophytes may often already be present in a location when new spores arrive, thus causing newly produced gametophytes to become antheridiate. Thus for experimental studies of the mating systems of species in which an antheridiogen system is controlling sexuality in nature, and depending on the goals of a study, it is important to establish laboratory conditions that allow for effective antheridiogen-mediated interactions.

In conclusion, we found that for generally inferring mating systems operating in nature (i.e., relative likelihood of intra- vs. intergametophytic mating), agar-based laboratory studies of gametophytes would lead to the same conclusions as would observations of field-collected gametophytes in *Sadleria* spp. For detailed studies of gametophyte sexuality and development, however, an agar-based medium produced significantly different results than what we found among natural populations of gametophytes. Whenever an even-aged population is created in the laboratory, however, this may be true on any growth medium. Thus, as have others before us, we suggest caution in the use of agar as a growth medium, and laboratory conditions in general, for studies of fern gametophyte sexual development.

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Additional Support for Two Subgenera of *Anemia* (Schizaeaceae) from Data for the Chloroplast Intergenic Spacer Region *trnL-F* and Morphology

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Abstract.—An analysis of morphological data for 13 species with 33 characters and molecular data for 14 species from the chloroplast DNA intergenic spacer region *trnL-F* indicates that species of the genus *Anemia* fall into two well-supported subgenera, *Anemiorrhiza* and *Anemia*. In addition, one species of the genus *Mohria* appears to belong within *Anemia*. Although further study is required, these data support the relationships suggested by a previous study of fossil and extant representatives of the genus.

The fern genus *Anemia* Sw. (Schizaeaceae) comprises about 120 species distributed mainly within the tropics and subtropics. Most of the species are found within the New World, with only about 12 in Africa and one in India. No monograph of the whole genus has been produced, although subgenus *Coptophyllum* (Mickel, 1962), subgenus *Anemiorrhiza* (Mickel, 1981), and spores of subgenera *Coptophyllum* and *Anemia* (Hill, 1977, 1979) have been studied. Since those works were produced, several new species have been described (Mickel, 1982, 1984, 1985), and a study of some of the Cretaceous fossils within the genus completed (Skog, 1992). In addition, the spores of the family Schizaeaceae have been described for modern and fossil representatives (van Konijnenburg-van Cittert, 1991, 1992). Other fossil representatives of the family have been described from Mesozoic and Cenozoic time periods and are summarized in Skog (2001) and Collinson (2001). There is clearly a need for a new revision of the genus and integration of all morphological data from fossil and living species with data from new molecular studies. We began a collaborative study in 1999. This paper reports some results indicating that the chloroplast sequence data are consistent with the fossil phylogeny reported earlier by Skog (1992).

The Schizaeaceae, considered to be a basal family of leptosporangiate ferns, includes the genera *Lygodium*, *Schizaea*, *Actinostachys*, *Mohria*, and *Anemia*. Fossil records of the family extend back to the Jurassic (Skog, 2001). The position of this family is not clear; it generally falls among several clades, including the Hymenophyllaceae, Cyatheaceae, Schizaeaceae, Matoniaceae, aquatic ferns, and more derived ferns (Raubeson & Stein, 1995; Pryer et al., 1995; Pryer et al., 2001). However, there is strong support from

the morphological and molecular evidence to date that the genera within the Schizaeaceae form a well-supported clade (Pryer et al., 1995; Pryer, 1999; Wikström et al., 2000, pp. 149–150). A molecular study using the chloroplast gene *rbcL* has been done for the Schizaeaceae and this study included several species of *Anemia* (Wikström et al., 2000).

Material and Methods

Species from which DNA was isolated and sequenced, as well as the voucher information are presented in Table 1. Morphological characters (Table 2, 3) for the preliminary analysis were derived from the descriptions and characters cited in the literature (Mickel, 1962, 1967, 1981, 1982; Skog, 1992; Tryon & Tryon, 1982).

Three subgenera (*Anemiorrhiza*, *Coptophyllum*, and *Anemia*) were represented in the samples. Both herbarium specimens and living material dried in silica gel were sources for the DNA. More recent herbarium specimens provided sequence data, but many older specimens yielded little, if any, DNA. Other taxa included were one species of *Mohria*, which is often suggested as congeneric with *Anemia* (Mickel, 1962), two species of *Lygodium*, one species of *Schizaea*, and a species in the Hymenophyllaceae (*Cardiomanes reniforme*) as the outgroup. The Hymenophyllaceae is also considered fairly basal among the ferns and has been shown to be more basal than the Schizaeaceae in analyses using morphology and *rbcL* chloroplast data (Pryer et al., 1995, 2001).

Total DNA was extracted from 15–20 mg of leaf tissue dried in silica gel (then frozen in liquid nitrogen) with the DNEasy Plant Mini kit from Qiagen, following the manufacturer's protocol. Double-stranded DNA amplifications were performed in a 50 µl volume containing 5 µl $MgCl_2$, 4 µl dNTP, 5 µl buffer, 2 µl primers, 1 µl BSA, 5 µl DNA, 0.5 µl TAQ, and 25.5 µl distilled water to volume. Following an activation step of 10 min at 94°C for the enzyme, the PCR mixture underwent 35 cycles of 1 minute at 94°C, 30 seconds at 50°C, 48°C or 54°C, and 72°C for 90 seconds followed by 72°C for 7 minutes before being held at 4°C. A few species were sequenced for the cpDNA gene *rbcL* using the primers previously published for fern sequences (Hasebe et al., 1995). The *trnL* (UAA)-*trnF* (GAA) intergenic spacer was amplified and sequenced with universal primers "e" and "f" (Taberlet et al., 1991).

Sequence data were generated for both strands of PEG (polyethylene glycol) purified PCR product using the ABI PRISM dye terminator cycle sequencing ready reaction kit in 20 µl volume containing 4 µl Big Dye terminator, 3.2 µl 1% primer, 2 µl DNA, 2 µl 5µ buffer, and 8.8 µl distilled water. Sequencing cycles followed the protocol for the ABI 377 PRISM Sequencer (Applied Biosystems, Inc.): 4 min at 96°C; then 25 cycles of 10 sec at 95°C, 0.5 sec at 50°C, 4 min at 60°C; followed by 1 min at 96°C; and then 4°C as the holding temperature. Following this step, excess dye terminators were removed by a spin column purification. Sequencing reactions were electrophoresed for 18 hours on an ABI PRISM 377 DNA sequencer in a

TABLE 1. List of species (with subgenera of *Anemia* in paraentheses), voucher specimens, and localities for the material used in the molecular analysis.

Species (Subgenus)	Specimen	Locality
Anemia adiantifolia (*Anemiorrhiza*) (L.) Sw.	Bradley 30582, GMUF	Bahamas, Andros Isl.
A. cicutaria (*Anemiorrhiza*) Kunze ex. Spreng.	Bradley 30691, GMUF	Bahamas, Andros Isl.
A. jaliscana (*Anemia*) Maxon	Mickel 1689, NY	Mexico, Jalisco
A. munchii (*Anemia*) Christ	Mickel 6874, NY	Mexico, Oaxaca
A. semihirsuta (*Anemia*) Mickel	Mickel 1120, NY	Mexico, Oaxaca
A. hirsuta (*Anemia*) (L.) Sw.	Prado 1064, NY	Brazil, São Paulo
A. phyllitidis (*Anemia*) (L.) Sw.	Mickel 1121, NY	Mexico, Oaxaca
A. underwoodiana (*Anemia*) Maxon	Greuter & Rankin 24997, B	Dominican Republic
A. villosa (*Coptophyllum*) Humb. & Bonpl. ex Willd.	Salino 1771, NY	Brazil, São Paulo
Mohria cafforum (L.) Desv.	NYBG 136/97 (living coll.)	South Africa
Lygodium microphyllum (Cav.) R. Br.	NYBG 1245/89B (living coll.)	unknown
L. flexuosum (L.) Sw.	NYBG 1281/76B (living coll.)	unknown
Schizaea elegans (Vahl) Sw.	H. Tuomisto 12744, TUR	Peru, Loreto
Cardiomanes reniforme (Forst.) C. Presl	A.R. Smith 2606, UC	New Zealand, No. Isl.

Long Ranger gel. The resulting chromatograms were edited with Sequencher version 3.1 (Gene Codes, Inc.) and the final consensus sequences were exported for alignment to Clustal X. All sequences were aligned manually with the aid of Se-Al version 1.0a1 (Rambaut, 1996) multiple sequence editor following initial alignment in the program Clustal X. Alignment of the *rbcL* data was guided by the sequences of Pryer et al. (1995). The *trnL-F* sequences were aligned by eye within the genus and between genera using the editing program Se-Al. Sequences were submitted to GenBank (Accession numbers AF448922-AF448935), and the alignment used in phylogenetic analyses will be posted at www.lms.si.edu.

All phylogenetic reconstruction analyses were conducted using version 4.08b of PAUP* (Swofford, 2001). Phylogenetic reconstruction under maximum parsimony was conducted using both the heuristic search algorithm with TBR branch-swapping, MULPARS and ACCTRAN options active, as well as the Branch and Bound search option in PAUP*. For molecular data, characters were assigned equal weights at all nucleotide positions; for morphological characters, equal weighting was also used. Robustness of cladistic linkages was evaluated with 1000 bootstrap replicates.

RESULTS

Results from the few species sequenced for *rbcL* were in agreement with the study done by Wikström et al. (2000). After their abstract was published, we dropped this gene from our study and accepted their discussion of a phylogeny based on that gene. Their topology has not yet been published. The plastid DNA sequence *trnL-F* was chosen because it has been shown to have

TABLE 2. Character names and states used in the morphological analysis.

1. rhizome habit
   0: compact, horizontal
   1: creeping
   2: compact, upright
2. stele
   0: dictyostele
   1: solenostele
3. axillary pockets
   0: absent
   1: present
4. arrangement of leaves
   0: polystichous
   1: distichous
5. stipe color
   0: stramineous
   1: variable or med. brown
6. trichomes on rhizome
   0: scales
   1: hairs long (4–10 mm)
   2: hairs short (1–3 mm)
7. rhizome trichome color
   0: orange
   1: dark brown or maroon
   2: yellowish brown
8. stipe size
   0: slender (0.6 mm)
   1: medium (1.0 mm)
   2: stout (2–3 mm)
9. frond division
   0: once pinnate
   1: pinnate-pinnatifid
   2: bipinnate
   3: dichotomous
10. pinna number
   0: several pairs (4–8)
   1: many (10–15+)
   2: none
11. pinna base shape
   0: truncate
   1: cuneate
12. epidermal cell shape
   0: isodiametric
   1: elongate
13. stomata
   0: attached
   1: floating
14. laminar hairs
   0: uni- or multicellular
   1: multicellular
   2: unicellular
15. laminar trichomes
   0: glandular
   1: nonglandular, pointed
   2: nonglandular, broad
16. fertile pinna position
   0: horizontal
   1: erect
17. fertile pinna placement
   0: remote from sterile
   1: approximate
18. fertile pinna length
   0: shorter than sterile blade
   1: exceeding sterile blade
   2: ca. equal to sterile blade
19. fertile pinna differentiation
   0: undifferentiated
   1: basal pair differentiated
   2: fronds fully dimorphic
   3: ≥2 pairs differentiated
20. fertile pinna arrangement
   0: opposite
   1: subopposite
   2: alternate
21. fertile pinna insertion
   0: sessile
   1: stalked
22. veins
   0: free
   1: anastomosing
23. sporangia shape
   0: oval, oblong
   1: spherical
24. apical plate cells
   0: thick-walled
   1: thin-walled
25. spore shape
   0: tetrahedral
   1: round
   2: bilateral, elongate
   3: tetraglobose (rounded)
26. spore ornamentation
   0: narrow sulci, wide muri
   1: wide sulci, narrow muri
   2: granulate
27. groove ornamentation
   0: smooth
   1: granulate
28. ridge ornamentation
   0: smooth
   1: granulate
   2: spinose
29. spore size
   0: <50μm
   1: 50–60
   2: 60–80
   3: >85
30. spore ridge position
   0: parallel, free
   1: coarsely reticulate
   2: finely reticulate
31. fertile pinnules on pinna
   0: all fertile
   1: intermixed with sterile
   2: at tips of sterile
32. Stipe shape
   0: terete
   1: somewhat flattened
   2: flattened
33. indument on stipe
   0: hairs scattered
   1: abundant orange hairs
   2: abundant white hairs
   3: abundant stiff black hairs
   4: subglabrous

relatively high and even substitution rates among the plastid loci within angiosperms (Richardson et al., 2000) and it has been successfully used for phylogenetic analyses among species within a genus of angiosperms (Molvray et al., 1999). It had not been used for many analyses within the ferns, but has been used for a study in a eusporangiate fern family (Hauk et al., 1996), and we have had some success in a study of the Osmundaceae (in progress). Within that region, only sequences between the "e" and "f"

TABLE 3. Character state matrix for ten species of *Anemia* and one each of *Mohria*, *Actiniostachys*, and *Schizaea*. Character coding is given in Table 2. Species abbreviations for *Anemia* use the subgeneric designations given in Table 1.

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
Ar. adiantifolia	1	1	0	1	0	2	1	0	2	0	0	1	0	2	1	1	1	2	1	0	1	0	1	0	1	1	1	0	1	0	0	0	0
Ar. cicutaria	1	1	0	1	0	2	1	0	2	0	0	1	0	2	1	1	0	1	2	2	1	0	1	0	1	1	0	0	1	2	0	0	0
Ar. wrightii	1	1	0	1	0	2	1	0	1	0	0	1	1	1	1	1	0	1	2	1	0	0	1	0	1	1	0	0	2	1	0	?	?
An. hirsuta	0	0	1	0	0	1	2	0	1	1	0	?	1	1	0	1	1	1	1	0	1	1	1	1	0	1	0	1	2	0	0	0	4
An. munchii	0	0	1	0	0	1	2	0	0	0	0	?	1	1	0	1	1	?	1	0	1	1	?	1	?	1	?	2	2	?	0	?	2
An. phyllitidis	0	0	1	0	0	2	0	0	0	0	0	?	1	1	1	1	1	1	1	0	1	1	1	1	0	1	?	2	2	?	0	1	2
An. underwoodiana	0	0	1	0	0	1	0	0	0	0	1	?	1	1	0	1	1	1	1	0	1	1	1	1	0	1	0	2	0	0	0	1	1
Ac. villosa	2	0	1	0	0	1	0/1	2	1	1	0	?	1	0	0	1	0/1	0/1	1	0	1	0	0	0	3	0	0	0	3	0	0	0	2
An. jaliscana	0	0	1	0	0	1	2	0	0	0	0	?	1	1	0	1	1	?	1	0	?	0	0	0	?	0	?	2	2	?	0	0	4
An. semihirsuta	0	0	0	0	0	1	0	1	0	0	1	?	1	0	0	1	1	1	1	1	1	1	0	0	1	1	0	0	2	1	0	1	0
Mo. cafforum	0	0	0	0	0	0	0	0	1	0	?	0	0	0	2	0	0	?	0	?	?	0	0	0	3	0	0	0	3	0	1	?	?
Sc. elegans	0	?	0	0	1	1	2	?	3	2	1	?	0	1	1	1	1	2	3	?	1	0	0	?	2	2	1	?	0	?	2	2	0
As. pennula	1	?	0	0	1	1	1	?	3	2	1	?	0	1	?	1	1	0	2	?	1	0	0	?	2	0	1	1	0	1	2	1	3

primers were amplified successfully. The region amplified between primers "c" and "d" in all angiosperms studied in the Laboratory for Molecular Systematics (Smithsonian Institution) to date did not work, even with optimization attempts using a Stratogene Robocycler. We suspect that either an accelerated rate of evolution in that subregion of the cpDNA has resulted in the primers' failing to anneal or that the inversion and rearrangements of the chloroplast genome, reported for ferns by Raubeson and Stein (1995), will account for the amplification difficulties. The latter may be more probable, as we successfully amplified the *trnL-F* "c-d" region for the Osmundaceae (Bauder, Skog & Zimmer, unpubl.), but not for the higher fern genus *Elaphoglossum* (Skog et al., 2001, p. 87).

Within the Schizaeaceae, the *trnL* "e-f" spacers had a GC content from 36–40%; the outgroup *Cardiomanes* GC content was 32%. These numbers were similar to those obtained in Zimmer's previous work on the basal angiosperm families Winteraceae and Canellaceae (Karol et al., 2000). For the taxa studied, there was significant length variation among the sequences.

Within the Schizaeaceae, the *trnL* sequences ranged in length from 387 base pairs (bp) (*Schizaea elegans*) up to 525 bp (*Anemia cicutaria*); in the outgroup *Cardiomanes reniforme*, the *trnL* region sequenced was 483 bp long. Within previously described subgenera of *Anemia*, the range of spacer lengths was much more limited. For subg. *Anemia*, spacers were 494–506 bp long; for subg. *Coptophyllum* the single *trnL* region sequenced from *Anemia villosa* was 515 bp long; for the two species of subg. *Anemiorrhiza*, *A. adiantifolia* at 524 bp was a single nucleotide shorter than *A. cicutaria*. *Mohria cafforum*, sometimes proposed as being nested within *Anemia*, had a *trnL* "e-f" region of 493 bp. The two *Lygodium* species were 481 and 489 bp long.

Given the degree of variation in spacer lengths among the *Anemia* species and among taxa at the family level in the Schizaeaceae, we had to infer a number of indel events when aligning the sequences. The overall alignment required gaps to be inserted into the raw sequences to a final length of 803 positions. As would be expected from the raw sequence lengths, most of the gaps that were inferred differentiated *Anemia* (including *Mohria*) from the other two genera of Schizaeaceae and that family from the outgroup *Cardiomanes*. Few of the gaps were autapomorphic. Many of the larger gaps separated genera, while those within *Anemia* were shorter in length. Refinement of the alignment used in subsequent phylogenetic inference will benefit from a much broader sampling of taxa across *Anemia* and within *Schizaea* and *Lygodium*. However, the relatively lower degree of length variation within and between *Anemia* and *Lygodium* suggests that the general phylogenetic patterns presented below will not be strongly affected by increased taxon sampling.

In the raw aligned data set for just the *trnL*"e-f" region, 372 characters were constant, 180 characters were parsimony-uninformative, and 251 characters were parsimony-informative. These numbers can be contrasted with those for the angiosperm family Winteraceae, where the entire *trnL* "c-d" and "e-f" regions could be aligned with Canellaceae outgroup genera for a total length of 990 characters and where 913 characters were constant, 60 characters were

parsimony uninformative, and only 17 were parsimony-informative (Karol et al., 2000). The high degree of length variation and large number of parsimony informative characters seen with just the smaller PCR product from the *trnL* region suggest that additional chloroplast spacer regions will be extremely useful in delineating species relationships in the Schizaeaceae.

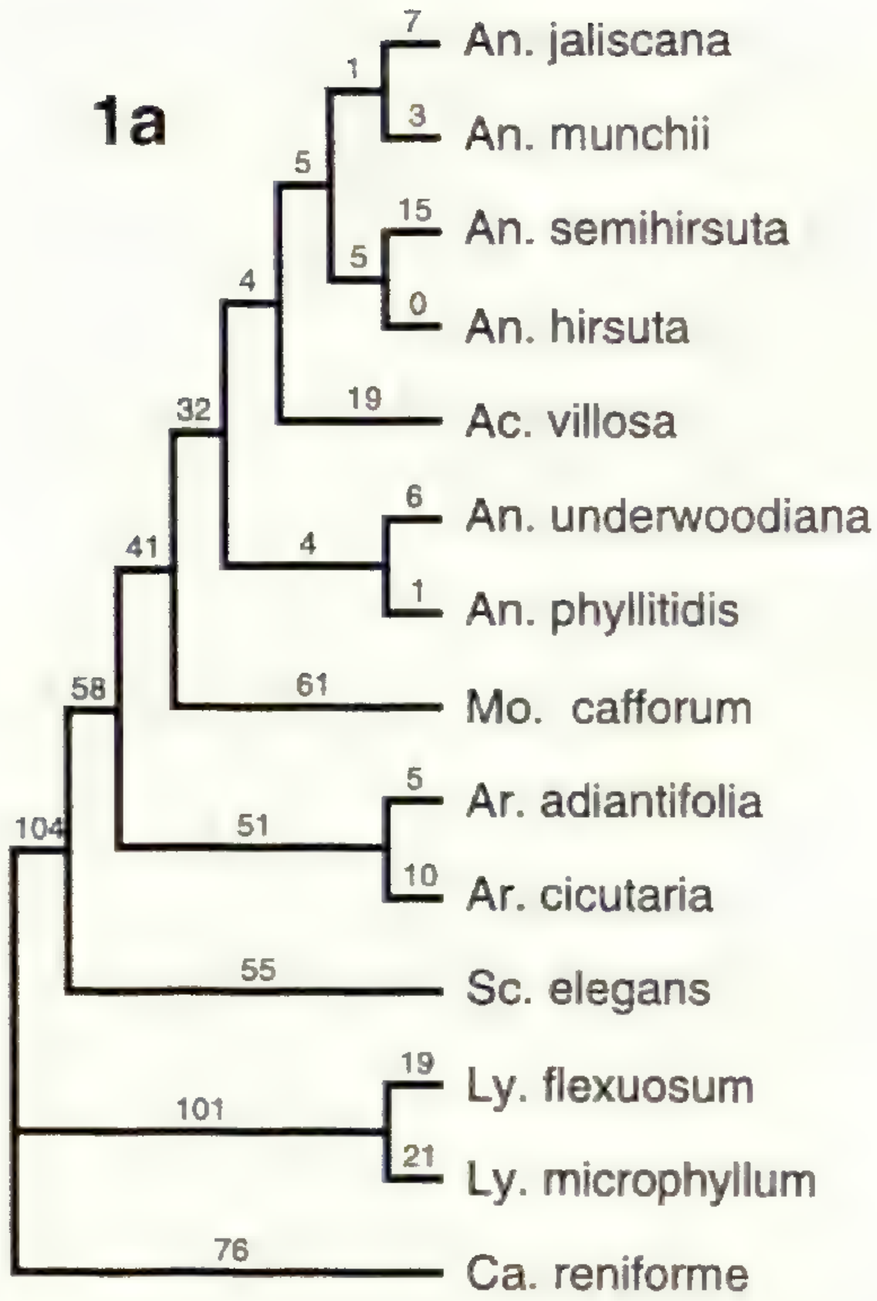
Both the Heuristic search and the Branch and Bound search options in PAUP* produced one most parsimonius tree of length 704 steps, with a consistency index of 0.875 and a retention index of 0.804. This tree is presented in Figure 1, with branch lengths given in Figure 1a and bootstrap values in Figure 1b. The same topology is obtained when the gap characters were ignored, *Cardiomanes* was excluded from the analysis, and *Schizaea* and *Lygodium* were set as outgroups. In addition, we used our data for *trnL* from the various genera (set as outgroups) within the Osmundaceae (*Osmunda, Todea, Plenasium, Leptopteris*) in the analysis and obtained the same topology for the tree. Maximum likelihood analyses of the data, using either the default options in PAUP* (Tn/Tv ratio = 2) or a more complex HKY85+G+I model, where base frequencies and the proportion of invariant sites were calculated from the data, also yielded a single tree with a topology identical to that obtained in the parsimony analysis (data not shown).

The morphological analysis is incomplete. To date, 56 species of the genus have been coded for 64 characters; however, the data were pruned to only 13 species and 33 characters for this paper (Tables 2, 3), which were obtained from species descriptions in the literature (Mickel, 1962, 1967, 1981, 1982; Skog, 1992; Tryon & Tryon, 1982). These 13 species were chosen because we also had material for DNA extraction. We present a brief outline of the results from the pruned morphological analysis, as it suggests several interesting hypotheses to be tested (Figure 2). A single most parsimonious tree was obtained from this reduced data set, shown in Figure 2a with branch lengths and Figure 2b with bootstrap values. Eighteen characters were synapomorphic for various clades. Subgenus *Anemiorrhiza* forms a distinct clade within the genus (66% bootstrap). The traditional subgenera *Anemia* and *Coptophyllum* cluster together with 52% support for a clade of these subgenera and *Mohria*. There is strong support for *Mohria* to be included within the genus *Anemia* (98%). The one species of *Mohria* and the one species of subg. *Coptophyllum* form a clade with 69% support. Obviously the expanded morphological data set is necessary, as more species of subg. *Coptophyllum* and *Mohria* are needed to determine if the relationship receives continued support from both morphological and molecular data. At the moment, only morphological data indicate this relationship. If these relationships are to be resolved, additional molecular data is also needed for the rest of the species of *Mohria* and additional species of *Anemia*.

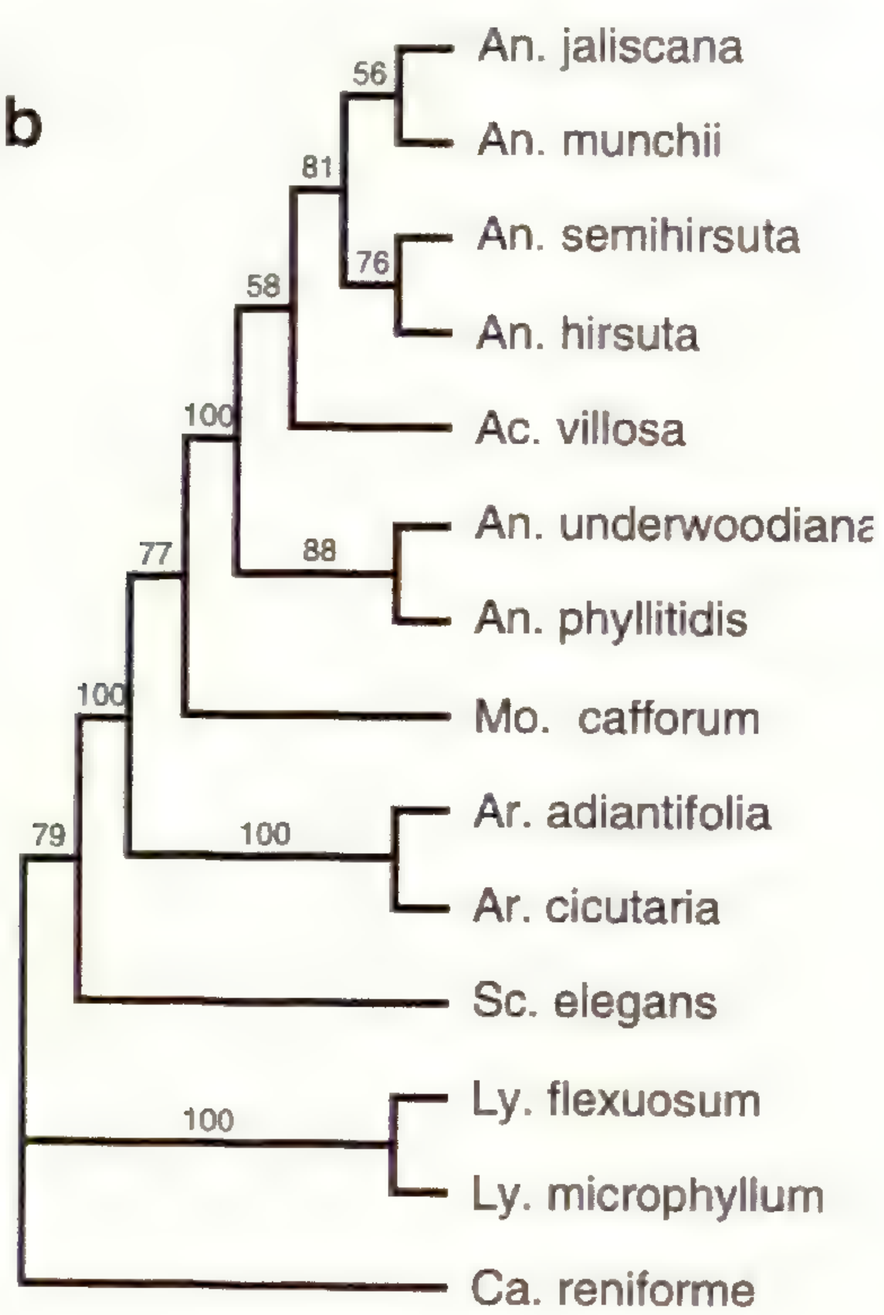
Discussion

In their published abstract, Wikström et al. (2000) noted that a maximum parsimony analysis of 30 living species of Schizaeaceae indicated that *Schi-*

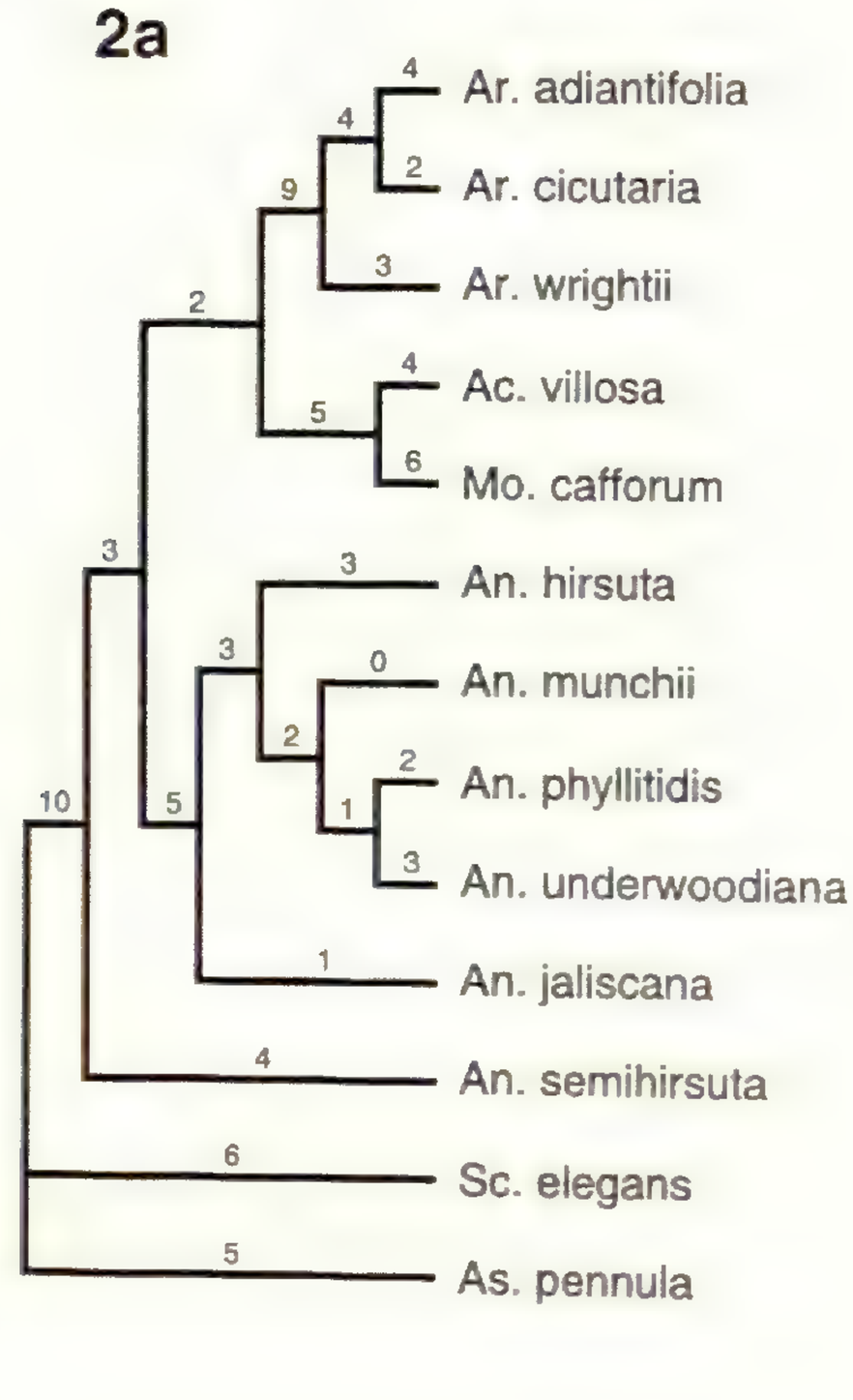
1a


An. jaliscana
An. munchii
An. semihirsuta
An. hirsuta
Ac. villosa
An. underwoodiana
An. phyllitidis
Mo. cafforum
Ar. adiantifolia
Ar. cicutaria
Sc. elegans
Ly. flexuosum
Ly. microphyllum
Ca. reniforme

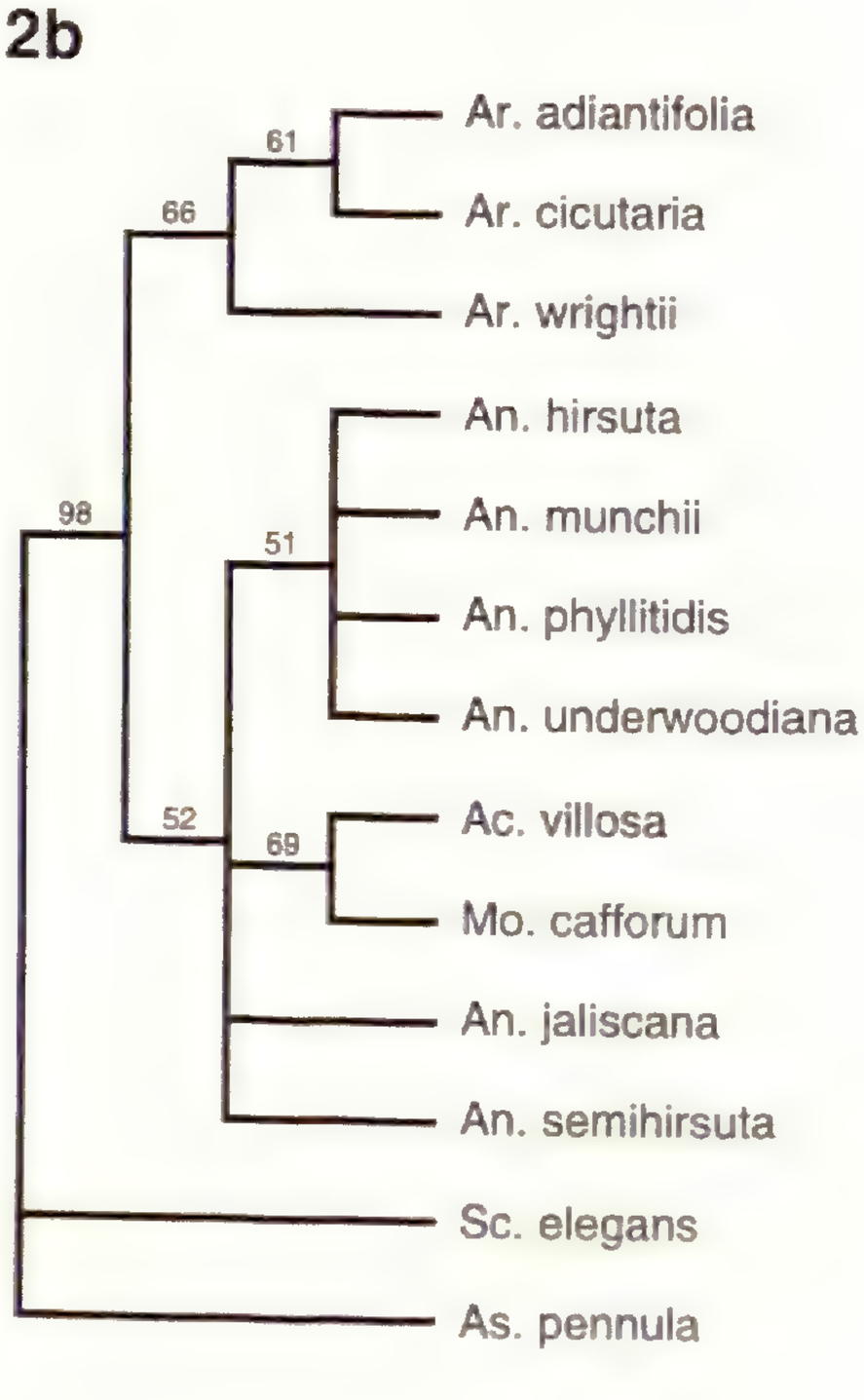
1b


An. jaliscana
An. munchii
An. semihirsuta
An. hirsuta
Ac. villosa
An. underwoodiana
An. phyllitidis
Mo. cafforum
Ar. adiantifolia
Ar. cicutaria
Sc. elegans
Ly. flexuosum
Ly. microphyllum
Ca. reniforme

2a


Ar. adiantifolia
Ar. cicutaria
Ar. wrightii
Ac. villosa
Mo. cafforum
An. hirsuta
An. munchii
An. phyllitidis
An. underwoodiana
An. jaliscana
An. semihirsuta
Sc. elegans
As. pennula

2b


Ar. adiantifolia
Ar. cicutaria
Ar. wrightii
An. hirsuta
An. munchii
An. phyllitidis
An. underwoodiana
Ac. villosa
Mo. cafforum
An. jaliscana
An. semihirsuta
Sc. elegans
As. pennula

zaea and *Lygodium* were monophyletic. *Anemia* was paraphyletic to *Mohria*, as was subg. *Coptophyllum* to subg. *Anemia*. They noted that *Anemiorrhiza* was a sister-group to a clade with all the other species of *Anemia* and *Mohria*. They stated that within the family there was a long branch leading to the species of *Lygodium*. The outgroup used in their analysis was not mentioned. The actual tree topology from their study has not yet been published and was not included in their abstract.

Our data are not completely consistent with their study. The *trnL* tree agrees with Wikström et al. (2000) in the placement of taxa within *Anemia* (Figure 1). However, *Schizaea* forms a strongly supported monophyletic clade with *Anemia* (79% bootstrap), and only the two *Lygodium* species are on a separate long branch relative to the outgroup *Cardiomanes*. *Mohria* clearly falls within *Anemia* (100% support), and basal to the clade of subgenera *Anemia* and *Coptophyllum* (77% support). The single species of subg. *Coptophyllum* falls within subg. *Anemia*, and this clade plus *Mohria* are sister to subg. *Anemiorrhiza*, which has 100% support on our tree. We believe that subg. *Coptophyllum* and subg. *Anemia* should be combined into a single subg. *Anemia*. There are no good morphological characters to support the separation of these two subgenera, and the molecular data do not support their separation either. Furthermore, we suspect that when additional species and characters are included in the analysis, subg. *Coptophyllum* and *Anemia* will form a strong single clade within the genus. There is, however, strong support for subg. *Anemiorrhiza* as a monophyletic taxon within an expanded genus *Anemia* (including *Mohria*).

As Mickel (1962) noted, *Mohria* is congeneric with *Anemia*. Traditionally the separation of these two genera was based mainly on the possession of scales by *Mohria*, but, as noted by Skog (1992), these scales have filiform tips similar to the trichomes found in *Anemia* and the trichomes on the leaves of the two genera are identical. *Mohria* bears sporangia on all pinnae of the fertile frond, but the confinement of the fertile pinnae to the basal pair is not always definitive. Some species of *Anemia* have dimorphic fronds (Mickel, 1984). According to van Konijnenburg-van Cittert (1991, 1992), the spores of *Mohria* show a hollow area in the ridges of the exospore that is not found in

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FIG. 1a. The single most parsimonious tree derived from DNA sequences *trnL* "e-f" region using the branch-and-bound option under maximum parsimony settings in PAUP*. Branch lengths are given above the lines leading to each lineage. Fig. 1b. Bootstrap consensus tree for the molecular data; bootstrap support values above 50% are given above the lines leading to the lineages. Species are those listed in Table 1. The abbreviations are: Ac = *Anemia* subg. *Coptophyllum*, An = *Anemia* subg. *Anemia*, Ar = *Anemia* subg. *Anemiorrhiza*, Mo = *Mohria*, Sc = *Schizaea*, Ly = *Lygodium*, and Ca = *Cardiomanes*.

FIG. 2a. The most parsimonious tree based on the preliminary morphological data set, using the same options as above. Branch lengths are given above the lines leading to each lineage. Fig. 2b. Bootstrap consensus tree for the morphology data set. Bootstrap values are given above the line. The abbreviations are those used in Figure 1, plus As = *Actinostachys*.

the spores of *Anemia* subg. *Coptophyllum* and *Anemia*. She also noted (1992) that hollow ridges occur in subg. *Anemiorrhiza*. Mickel (1962, Plate IV) discussed and illustrated three types of ridge structure: a solid ridge with no internal differentiation, a medulla within the ridge containing a spongy network, or a simple medulla, which may be a small portion of the ridge as in *Mohria* and in some species of *Anemia*. We see few substantial arguments to maintain *Mohria* and *Anemia* as separate genera, although we await the outcome of expanded analyses that include more than a single species of *Mohria*.

Hill (1977, 1979) suggested that spore morphology might be an important character within *Anemia* because the spore morphology was a conservative character. Even in our preliminary analysis of morphological characters, we see that the spore morphology of ridges, grooves, and bacculae will help to delimit taxa, and that more species require examination for the critical characteristics of the spores. Some characters that should be included are, for example, elaborations at the angles of the spore outline, orientation of the ridges, width of the striations, cross ridges between the ridges, and ornamentation on the ridges and between the ridges.

The *trnL* data support the phylogeny previously inferred from the morphological study of fossil and modern species (Skog, 1992). No outgroup was used in that analysis. At the time, no fossil representatives were known for the Hymenophyllaceae earlier than the appearance of the Schizaeaceae; characters from the fossils known were few, leading to a matrix with many missing characters; and relationships within the primitive fern families were not stable or easy to discern. However, the analysis of the species within *Anemia* and the fossils attributed to the genus or closely aligned to it indicates that there is strong support for the subg. *Anemiorrhiza*, that subg. *Coptophyllum* is paraphyletic to subg. *Anemia*, that these form a sister group to *Anemiorrhiza*, and that *Mohria* and two fossil species form another clade that is not placed consistently in either of the other two clades and was better supported as a third group (Skog, 1992).

Interesting questions remain concerning *Anemia*. Subgenus *Anemiorrhiza* is consistently diploid, whereas the other subgenera have various levels of polyploidy and several hybrids have been reported (Mickel, 1982). It may be that, contrary to previous hypotheses (Mickel, 1962), species within subg. *Coptophyllum* are not the most primitive in the genus. Extant species of *Anemia* are most numerous in Mexico and Brazil, and these areas also have the greatest current morphological diversity for the genus. However, the family Schizaeaceae first appears in the northern hemisphere in the Mesozoic, as does *Anemia* (Skog, 2001), suggesting biogeographic questions might be addressed when the phylogeny of the genus is better known. There are also many morphological characteristics of fertile pinnae that might be addressed. For example, the fertile pinnae of *Anemia* are most commonly basal and extended upright. However, there are some species with dimorphic fronds, and several species, such as *A. colimensis* and *A. salvadorensis* (Mickel, 1967, 1984), have nonextended fertile pinnae. These latter species appear to be

morphologically intermediate between the fossil species, which had complete fertile fronds, interspersed fertile pinnules, or basal fertile pinnules. Whether these extant species with nonextended fertile pinnae form a group or are dispersed throughout the genus will be of benefit in the understanding of the development of the fertile fronds and the fertile pinnae. In our preliminary morphological trees for the expanded data set, species with nonextended fertile pinnae are currently scattered throughout the tree.

Based upon our data, we support the phylogeny of *Anemia* suggested previously by fossil data (Skog, 1992) and *rbcL* data (Wikström et al., 2000, pp. 149–150). We support only two of the three subgenera of the current genus *Anemia*: *Anemiorrhiza* and *Anemia* (including subg. *Coptophyllum*). We believe that *Mohria* should be placed in *Anemia*.

ACKNOWLEDGEMENTS

This paper is dedicated to the memory of Warren Herb Wagner (1920–2000). Professor Wagner taught Skog the modern ferns and encouraged her study of fossil ferns. He was the mentor, advisor, and friend of Mickel. We also thank Youngbae Suh for DNA sequencing instruction and Molly Nepokroeff and Ken Karol for advice on sequence editing and alignment, as well as phylogenetic analyses. We are indebted to reviewers Alan Smith, Don Farrar, and Tom Ranker for their helpful improvements to the manuscript.

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Intrafamilial Relationships of the Thelypteroid Ferns (Thelypteridaceae)

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ABSTRACT.—Data from three chloroplast genes (*rps4* + *trnS* spacer, + *trnL* spacer; 1350 base pairs) for 27 of the recognized segregates show the Thelypteridaceae to be monophyletic and sister to an unresolved alliance of blechnoid, athyrioid, onocleoid, and woodsioid ferns. The family comprises two primary lineages, one phegopteroid, the other thelypteroid (including cyclosoroid). The phegopteroid lineage (*Macrothelypteris*, *Pseudophegopteris*, and *Phegopteris*) includes those elements that are the most dissected, lack adaxial grooves on the frond axes, and are generally morphologically the most distinct elements within the family. Within the thelypteroid-cyclosoroid lineage, three predominantly north-temperate subgroups, including *Thelypteris s.s.*, form a free-veined clade that is in turn sister to the rest of the family. All segregates possessing $x=36$ (*Cyclosorus sensu* Smith, with predominantly anastomosing veins) form a strongly supported clade. Those groups with dysploid base chromosome numbers ($x=27$, 29, 30, 31, 32, 33, 34, 35) form a series of smaller clades basal to *Cyclosorus s.l.* Although our sampling is not yet sufficient to favor one classification over another, recognition of an intermediate number of genera may be the most reasonable taxonomic course.

Since its taxonomic separation from the dryopteroid ferns as a distinct group, about 60 years ago, Thelypteridaceae has been treated as a natural group comprising nearly 1000 mostly tropical species. Although generally recognized as a natural monophyletic group, there is a wide divergence of views about generic circumscription. Morton (1963) placed all species in a single genus *Thelypteris*. Ching (1963) outlined a classification that accepted 18 Asian genera, including *Hypodematium*, which is generally excluded from the family by other workers, even by Ching (1978). Ching (1978) later added two newly described genera to Thelypteridaceae: *Craspedosorus* (which we regard as a synonym of *Leptogramma*, often included in *Stegnogramma sensu* Iwatsuki, 1963); and *Trichoneuron* (regarded by us as belonging to *Lastreopsis*, a dryopteroid, definitely not thelypteroid). Both of these genera are monotypic and poorly known. Still later, Shing (1999), subsumed *Amphineuron* under *Cyclosorus s.l.* and removed *Trichoneuron* from the family. Iwatsuki (1964) recognized three genera in the family, *Stegnogramma* and *Meniscium* (each with four sections), and *Thelypteris*, the last comprising 14 subgenera and several additional sections; Iwatsuki (1964:23) regarded two of his subgenera of *Thelypteris* (*Haplodictyum* and *Cyrtomiopsis*) as probably "generically distinct." Holttum (1971, 1982) characterized 25 genera in the Old World but did not explicitly address New World groups. Pichi Sermolli (1977:335–337), largely following Holttum, accepted 32 genera. In the most recent classification, Smith (1990) adopted an intermediate view, recognizing

five genera. Most of the paleotropical segregate genera (Holttum, 1969, 1972, 1973a, 1974, 1975, 1976a, 1976b, 1977, 1981; Holttum & Grimes, 1979; Iwatsuki, 1963), and several of the neotropical ones (Maxon & Morton, 1938; Smith, 1971, 1980), have been recently revised or monographed, making this one of the best known fern families morphologically, cytologically, and distributionally. Little, however, is known about relationships among these segregates.

The goals of this work are to provide a phylogenetic hypothesis for the Thelypteridaceae based on molecular evidence from the 25–30 groups commonly recognized within the family. A second goal is to confirm or refute the hypothesis of monophyly for the family. We make no attempt in this paper to incorporate a rigorous morphological data set to contrast with the molecular data set, although we find it useful, and we hope informative, to comment on certain characters that are often used to distinguish genera or groups of genera, in light of the molecular results. In this paper, we choose to concentrate on the higher-level relationships in the family, and this initial approach necessarily involves only one or, in a few cases, two species per group (genus, subgenus, section). A study directed to the generic subdivision of the family would need to incorporate a minimum of two and ideally at least three species per group, so as to address the monophyly of the individual genera, subgenera, or sections (depending on classification employed).

Materials and Methods

Taxon Sampling.—As a basis for sampling, we used the classification of Holttum (1971), as modified by Holttum (1982) for Old World groups. For New World groups, we sampled from groups recognized by Morton (1963). A total of 30 ingroup taxa were sampled (Table 1). These represent 19 of the 22 Malesian genera recognized by Holttum (1982; only *Ampelopteris*, *Amphineuron*, and *Cyclogramma* are missing from our analysis), 25 of the 32 genera of Thelypteridaceae recognized by Pichi Sermolli (1977; only *Ampelopteris*, *Amphineuron*, *Cyclogramma*, *Glaphyropteris*, *Haplodictyum*, *Menisorus*, and *Stegnogramma s.s.* are missing), and 13 of the 20 Chinese genera recognized by Ching (1978; only *Ampelopteris*, *Amphineuron*, *Craspedosorus* (regarded by us as a synonym of *Leptogramma*), *Cyclogramma*, *Mesopteris* (regarded by us as a synonym of *Sphaerostephanos*), *Stegnogramma s.s.*, and *Trichoneuron* are missing). All neotropical groups, with the exception of *Glaphyropteris s.s.* (which we regard as a subgroup of *Steiropteris*; Smith, 1980), were also sampled. Recent phylogenetic analyses of the Polypodiales (higher leptosporangiate ferns) by Hasebe et al. (1995) and by Cranfill (unpubl. data) indicate that the thelypteroid ferns belong to this order, and that the most closely related groups (families in some classifications) are an unresolved alliance of blechnoid, athyrioid, deparioid, onocleoid, and woodsioid ferns. Sixteen representatives from these families were used as outgroups.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING.—We utilized silica gel-dried leaf material and, in a few cases, leaf material removed from herbarium specimens as sources for genomic DNA, which was extracted using DNEasy Plant Mini DNA extraction kits from Qiagen Corporation. Amplification was performed using Amplitaq Gold DNA taq polymerase produced by Perkin Elmer Corporation. Ongoing work by Cranfill previously demonstrated the phylogenetic utility of the chloroplast gene *rps4* (Cranfill, 2000a, 2000b) and the *trnS* and *trnL-F* intergenic spacer regions (Cranfill, unpubl.), and so data from these three markers were collected for phylogenetic analysis. We also considered the inclusion of *rbcL*, but chose to exclude it from our study for reasons of time and economics. A preliminary analysis showed that *rbcL* data provided more or less the same phylogenetic information at roughly the same taxonomic levels as *rps4*.

The *rps4* region was amplified using the primers provided by Nadot et al. (1995), yielding an amplicon of approximately 650 bp. The intergenic spacer region between *rps4* and *trnS* was amplified using the reverse compliment of Nadot et al. (1995), reverse *rps4* primer R1 and the novel reverse primer *trnS* R (5'-TAC-CGA-GGG-TTC-GAA-TC-3'), yielding an amplicon of approximately 400 bp. The intergenic spacer at the 3′ end of *trnL-F* was amplified using primers e and f from Taberlet et al. (1991), yielding an amplicon of approximately 350 bp. Amplification was accomplished using standard thermocycling protocols, with annealing temperatures of 48°C–54°C for *rps4* and 45°C–48°C for the two intergenic spacer regions, using an MJ Research PTC-200 Peltier thermal cycler. Direct sequencing of each PCR product was conducted in both directions for each marker using the BigDye cycle sequencing kits produced by Applied Biosystems Inc. (ABI) using an ABI PRISM 377 DNA automated sequencer in the Molecular Phylogenetics Laboratory of the University of California, Berkeley.

DNA sequences were manually aligned. Alignment of the coding *rps4* sequences was unambiguous. The two intergenic spacer regions were aligned with more difficulty, and all ambiguously aligned regions were removed from the analyses. Gaps were treated as missing, while unambiguous and phylogenetically informative indels were treated as binary characters and coded at the end of the data matrix.

Maximum parsimony analyses were performed with PAUP* (Swofford, 1999). Combinability of data from the three markers was investigated using the partition homogeneity test (Farris et al., 1995), as implemented in PAUP*, the results of which supported data combination. Heuristic searches were then conducted on the combined data set with the following options in effect: 100 random addition replicates, tree-bisection-reconnection, branch swapping, and steepest descent. The searches were repeated ten times and allowed to run to completion in order to recover the shortest trees. The resultant trees were rooted with appropriate outgroups identified previously in the *rbcL* phylogeny by Hasebe et al. (1995), and confirmed by Cranfill from an unpublished five-gene molecular phylogeny of the Polypodiales. In addition to computing a strict concensus of trees obtained from our searches, we

TABLE 1. Sources of material of ingroup and outgroup species providing *rps4*, *trnL* spacer, and *trnS* spacer sequences for this study. All sequence data reported in this paper are newly generated. Parenthetical RBC numbers are DNA extraction numbers.

Species	Locality	Voucher/Source	Herb.	GenBank rps4-trnS[1]	GenBank trnL spacer
Amauropelta oligocarpa (Willd.) Pic. Serm.	Costa Rica: San José: Las Nubes	U. C. Bot. Gard. 57.0002	UC	AF425162	AF425125
Chingia longissima (Brack.) Holttum	Fiji: Viti Levu: Tomaniivi (Mt. Victoria)	Game 99/270 (RBC 818)	UC	AF425163	AF425126
Christella arida (D. Don) Holttum	Malaysia	Cranfill BF-22 (RBC 718)	UC	AF425164	–
Christella hispidula (Decne.) C. F. Reed	cult., unknown source	N. Y. Bot. Gard. 477/77A = Cranfill *s.n.* (RBC 577)	UC	AF425165	AF425127
Christella (Pelazoneuron) augescens (Link) Pic. Serm.	U.S.A.: Florida: Greynolds Park, S end West Lake, Miami	U. C. Bot. Gard. 78.0268 = Cranfill *s.n.* (RBC 785)	UC	AF425166	AF425128
Coryphopteris seemannii Holttum	Fiji: Viti Levu: Tomaniivi (Mt. Victoria)	Game 95/147B (RBC 817)	UC	AF427096/ AF427097	AF425129
Cyclosorus interruptus (Willd.) H. Itô	cult. by B. Parris, New Zealand	Cranfill *s.n.* (RBC 707)	UC	AF425167	AF425130
Dictyocline griffithii T. Moore	Taiwan	Cranfill TW-004 (RBC 637)	UC	AF425168	AF425131
Glaphyropteridopsis erubescens (Hook.) Ching	Taiwan	Cranfill TW-197 (RBC 640)	UC	AF425169	AF425132
Goniopteris poiteana (Bory) Ching	Mexico: Oaxaca: near Tuxtepec	Mickel 5799 = U. C. Bot. Gard. 78.0266 (RBC 788)	NY	AF425170	AF425133
Leptogramma tottoides H. Itô	Taiwan	Cranfill TW-79 (RBC 638)	UC	AF425171	AF425134
Macrothelypteris torresiana (Gaudich.) Ching	New Zealand	Cranfill *s.n.* (RBC 673)	UC	AF425172	–
Meniscium sp.	Panama: Canal Zone: across street from Summit Zoo	A. Smith 2633 (from P. Hammond) (RBC 786)	UC	AF425173	AF425135

TABLE 1. Continued.

Species	Locality	Voucher/Source	Herb.	GenBank rps4-trnS[1]	GenBank trnL spacer
Mesophlebion crassifolium (Blume) Holttum	Malaysia	Cranfill BF-59 (RBC 703)	UC	AF425174	AF425136
Metathelypteris dayi (Bedd.) Holttum	Malaysia	Cranfill CH-35 (RBC 745)	UC	AF425175	AF425137
Nannothelypteris aoristisora (Harr.) Holttum	Philippines: Mt. Makiling, Laguna	Price 670 (RBC 826)	UC	AF425176	–
Oreopteris limbosperma (All.) Holub	Great Britain: South Somerset, 4 km SW of Nether Stowey	Crabbe et al. 11830 (RBC 825)	UC	AF425177	–
Parathelypteris nevadensis (Baker Holttum	U.S.A.: California: Plumas Co., ca. 7 mi SE of Keddia	U. C. Bot. Gard. 60.0707 (RBC 464)	JEPS	AF425178	AF425138
Phegopteris connectilis (Michx.) Watt	cult., Mickel garden, New York	Cranfill *s.n.* (RBC 575)	UC	AF425179	AF425139
Phegopteris decursivepinnata (H. C. Hall) Fée	cult., Unknown source	N. Y. Bot. Gard. 685/76 = Cranfill *s.n.* (RBC 576)	UC	AF425180	–
Plesioneuron archboldiae (Copel.) Holttum	Fiji: Viti Levu: Serua, between Waininggere and Waisese Creeks	A. C. Smith 9348 (RBC 829)	UC	AF425181	–
Pneumatopteris ecallosa (Holttum) Holttum	Malaysia	Cranfill CH-25 (RBC 639)	UC	AF425182	AF425140
Pronephrium simplex (Hook.) Holttum	China: Hong Kong: Victoria Peak, Victoria Isl.	U. C. Bot. Gard. 79.0293 (RBC 580)	UC	AF425183	AF425141
Pseudocyclosorus esquirolii (H. Christ) Ching	Taiwan	Cranfill TW-29 (RBC 713)	UC	AF425184	AF425142

TABLE 1. Continued.

Species	Locality	Voucher/Source	Herb.	GenBank rps4-trnS[1]	GenBank trnL spacer
Pseudophegopteris aurita (Hook.) Ching	cult., Parris garden, New Zealand, orig. from Japan	Cranfill *s.n.* (RBC 238)	UC	AF425185	–
Sphaerostephanos penniger (Hook.) Holttum	Malaysia: Bangi Fern Garden	Cranfill BF-24 (RBC 641)	UC	AF425186	AF425143
Sphaerostephanos taiwanensis (C. Chr.) Holttum ex Kuo	Taiwan: Taibei Botanical Garden	Cranfill TW-231 (RBC 709)	UC	AF425187	–
Steiropteris leprieurii (Hook.) Pic. Serm.	French Guiana: Montagnes de la Trinité, Bassin de la Mana	Granville 13264 (RBC 828)	UC	AF425188	–
Thelypteris palustris Schott	N. Y. Bot. Gard. (wild)	Cranfill *s.n.* (RBC 574)	UC	AF425189	AF425144
Trigonospora ciliata (Benth.) Holttum	cult., unknown source	N. Y. Bot. Gard. acc. = Cranfill *s.n.* (RBC 582)	UC	AF425190	AF425145
Outgroups					
Acystopteris japonica (Luerss.) Nakai	Taiwan	Cranfill *s.n.* (RBC 590)	UC	AF425150	AF425121
Asplenium cristatum Lam.	Costa Rica: Puntarenas: Las Alturas, 31 km from San Vito	U. C. Bot. Gard. 90.2234 = Cranfill *s.n.* (RBC 042)	UC	AF425146	–
Asplenium nidus L.	cult. origin	Cranfill *s.n.* (RBC 309)	UC	–	AF425118
Athyrium filix-femina (L.) Roth ex Mert. *s.l.*	cult., Mickel garden, New York	Cranfill *s.n.* (RBC 356)	UC	AF425152	–
Cystopteris protrusa (Weath.) Blasdell	cult., Mickel garden, New York	Cranfill *s.n.* (RBC 369)	UC	AF425148	AF425120
Deparia lancea (Thunb. ex Murray) Fraser-Jenk.	cult., source unknown, native to SE Asia	U. C. Bot. Gard. 71.0038 = Cranfill *s.n.* (RBC 011)	UC	AF425153	AF425123
Didymochlaena truncatula (Sw.) J. Sm.	cult., unknown source	Cranfill *s.n.* (RBC 432)	UC	AF425161	–

TABLE 1. Continued.

Species	Locality	Voucher/Source	Herb.	GenBank rps4-trnS[1]	GenBank trnL spacer
Gymnocarpium oyamense (Baker) Ching	cult., Parris garden, New Zealand, origin from Japan	Cranfill *s.n.* (RBC 240)	UC	AF425149	–
Homalosorus pycnocarpos (Spreng.) Pic. Serm.	cult., Mickel garden, New York	Cranfill *s.n.* (RBC 597)	UC	AF425154	AF425124
Hypodematium crenatum (Forssk.) Kuhn	Japan, sent by M. Kato	Hyashi *s.n.* (RBC 768)	TI	AF425151	AF425122
Lorinseria areolata (L.) C. Presl	U.S.A.: S. Carolina: Orange Co., along Rte. 176	U. C. Bot. Gard. 82.2087 = Cranfill *s.n.* (RBC 170)	–	AF425155	–
Matteuccia struthiopteris (L.) Tod.	cult., original source unknown	North Carolina Bot. Gard = Cranfill *s.n.* (RBC 460)	UC	AF425158	–
Onoclea sensibilis L.	U.S.A.: Massachusetts	Weed s.n. = U. C. Bot. Gard. 80.0321 (RBC 005)	UC	AF425159	–
Onocleopsis hintonii F. Ballard	Mexico: Oaxaca, sent by Gastony	Mickel *s.n.*	IND	AF425160	–
Sadleria cyatheoides Kaulf.	U. S.A.: Hawaii: Maui: between Olinda and Wai Kamoi stream	Ornduff 8506 = U. C. Bot. Gard. 78.0380 (RBC 003)	UC	AF425156	–
Stenochlaena milnei Underw.	Philippines ("New Guinea" on label): Samar, from spores	U.C. Bot. Gard. 55.0076 (RBC 0.37)	UC	AF425157	–
Woodsia polystichoides D. C. Eaton	cult., Mickel garden, New York	Cranfill *s.n.* (RBC 551)	UC	AF425147	AF425119

[1] *rps4–trnS* sequence data represent one contiguous sequence with *rps4* at the 5′ end and the *trnS* spacer at the 3′ end. Sequences for *Coryphopteris* are for *rps4* alone (AF427096), *trnS* spacer alone (AF427097), and *trnL* alone (AF425129).

also explored clade stability using bootstrap resampling (Felsenstein, 1985), as implemented in PAUP*.

Results

Of the 2174 characters, 658 were parsimony informative. Analysis of these sites yielded 26 equally parsimonius trees of 2496 steps, having a consistency index of 0.55, a retention index of 0.63, and a rescaled consistency index of 0.35. A representative most parsimonious tree is depicted in Figure 1, while the strict concensus is presented in Figure 2. These analyses show that the Thelypteridaceae is monophyletic with the exclusion of *Hypodematium*, which is more closely allied with the dryopteroid genus *Didymochlaena* than with any of the outgroup representatives of the athyrioid ferns. Although there is no bootstrap support, the Thelypteridaceae is resolved as sister to a clade comprising *Acystopteris*, *Cystopteris*, and *Gymnocarpium*, at the base of the athyrioid lineages.

Thelypteridaceae comprises two major lineages with high bootstrap support: a phegopteroid lineage that includes *Macrothelypteris*, *Pseudophegopteris*, and *Phegopteris*; and a thelypteroid lineage comprising the remaining clades (Fig. 2). *Thelypteris s.s.* is basal in the thelypteroid lineage, which includes two well supported subgroups, an amauropeltoid clade that comprises *Amauropelta*, *Parathelypteris*, *Coryphopteris*, and *Metathelypteris*, and a strongly supported cyclosoroid crown group of 17 genera (Fig. 2). Within these groups, several smaller clades received significant bootstrap support, including a clade comprising *Dictyocline* and *Leptogramma* (100%) and a clade comprising *Goniopteris* and *Christella augescens* (78%) (Fig. 1).

Discussion

Monophyly and Relationships of Thelypteridaceae.—Since its formal establishment in 1970, no one has seriously questioned the monophyly of Thelypteridaceae (excluding *Hypodematium*), on either morphological or molecular grounds. Even before then, Christensen (1907, 1913, 1920) recognized the relatedness of genera now included in the family, and Ching (1940) circumscribed the Thelypteridaceae in a way that closely approximates recent classifications, with minor exceptions. Prior to the work of Ching, most species now regarded as belonging in Thelypteridaceae had been included in the comprehensive genus *Dryopteris*, which is now known to be only remotely related. Ching (1940, 1963, 1978), and Holttum (1971, 1973b) effectively refuted the dryopteroid relationship of the thelypteroid ferns.

Heretofore, molecular analyses have incorporated only a handful of Thelypteridaceae. Of studies that included them at all, the most notable is by Hasebe et al. (1995), who sampled four representatives in their global analysis: *Amphineuron opulentum* (Kaulf.) Holttum, *Christella acuminata* (Houtt.) Holttum, *Parathelypteris beddomei* (Baker) Ching, and *Thelypteris palustris*

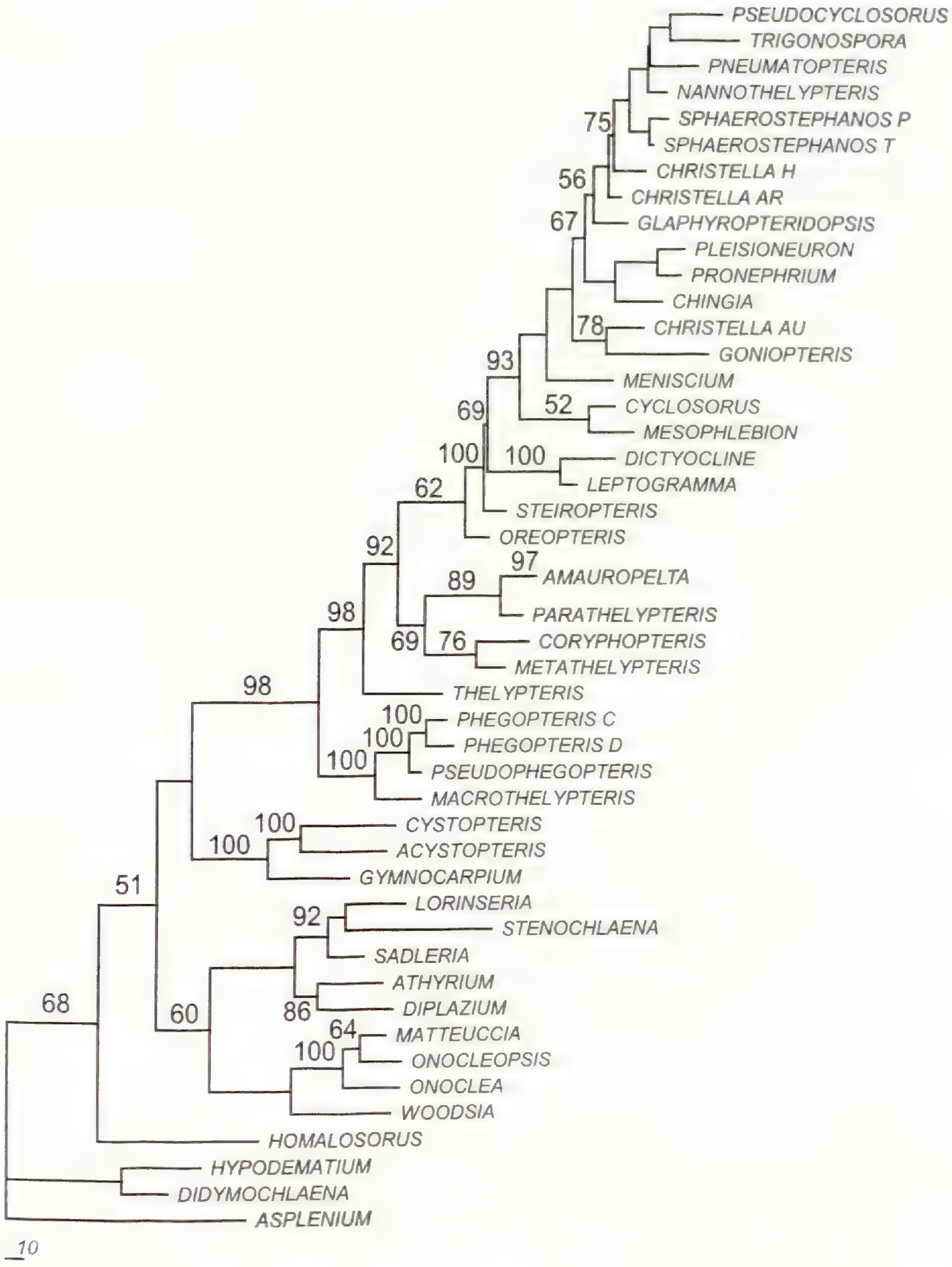


FIG. 1. One of 26 most parsimonious trees drawn as a phylogram, combined analysis utilizing the chloroplast genes *rps4*, *trnS* spacer, and *trnL* spacer. Branch lengths are proportional to the amount of change along each branch. Bootstrap support for clades, if greater than 50%, is indicated above branch nodes. Tree is 2496 steps; CI = 0.55; RC = 0.35; RI = 0.63. See Table 1 for full names of species and additional voucher data.

Schott (listed here under their segregate generic names). The first two of these species belong to cyclosoroid genera, the last two species to amauropeltoid genera or *Thelypteris s.s.* (Fig. 2). No representatives of the phegopteroid clade have been included in previous analyses, and no other global or family level molecular surveys have provided any additional evidence on relationships either outside or within the family. Our study clearly shows that Thelypteridaceae, in the sense used by nearly all modern authors, is monophyletic, with high (98%) bootstrap support (Fig. 1).

Holttum (1971) hypothesized a relationship between Thelypteridaceae and Cyatheaceae, a kinship that is now effectively refuted on the basis of ample morphological (Smith, 1990) and molecular evidence (Hasebe et al., 1995; Pryer et al., 1995; Wolf et al., 1999). Pichi Sermolli (1977) postulated a close relationship of Thelypteridaceae to Aspleniaceae, and indeed this relationship appears relatively close, although more remote than with several other large clades. Our analysis, and work by Cranfill (unpubl.), clearly show that the thelypteroid ferns are most closely related to a large terrestrial clade comprising the athyrioid, woodsioid, blechnoid, and onocleoid ferns (Fig. 1). The exact interrelationships of these immediate outgroups are still not well supported in our analysis or any other published molecular analysis.

INTRAFAMILIAL RELATIONSHIPS.—Using morphological and cytological evidence, several students of the family have postulated intrafamiliar relationships within Thelypteridaceae, most notably Loyal (1963), Smith (1971), and Pichi Sermolli (1977). All of these studies invoked morphological and cytological evidence, and offered somewhat different and conflicting pictures of relationships. Loyal (1963) depicted two main evolutionary lines within the family, one line with species groups having a chromosome base number of 36, with species having one or more pairs of veins from adjacent pinna segments united below the sinus. The second evolutionary line in Loyal's scheme comprised species mostly with dysploid (stepwise progression in the basic chromosome set) chromosome numbers (27 to 35) and free veins. Smith's (1971:46) scheme was an attempt to update Loyal's tree, and showed basically a free-veined evolutionary line, with the phegopteroid ferns terminating this branch, and three shorter anastomosing- or connivent-veined lines having $x=36$. These included an Old World line, a New World line, and *Stegnogramma s.l.*

Pichi Sermolli (1977:441) hypothesized a basal dichotomy in the family leading, along one line, to the evolution of several free-veined genera (phegopteroid ferns plus *Metathelypteris*), followed by other free-veined elements (*Parathelypteris*, *Coryphopteris*, *Oreopteris*, and *Thelypteris*), and ultimately giving rise to several free-veined and connivent-veined New World species groups (*Amauropelta*, *Steiropteris*, and *Glaphyropteris*). As a second evolutionary line, Pichi Sermolli derived all of the anastomosing-veined Old World genera. Although Holttum (1971, 1982) revised nearly all of the Old World genera, and commented extensively on relationships, he never presented a formal phylogenetic treatment. Holttum (1971) believed

that certain Old World cyclosoroid genera (*Pronephrium*, *Nannothelypteris*, *Stegnogramma*, *Sphaerostephanos*, and *Pneumatopteris*) formed a natural group. He also postulated that *Mesophlebion*, *Chingia*, and *Glaphyropteridopsis* were related, and that *Coryphopteris* and *Parathelypteris* formed an isolated group. According to Holttum (1982:386), *Cyclosorus s.s.* and *Ampelopteris* (with united veins) and *Thelypteris s.s.*(with free veins), all with wide distribution and similar aquatic habitat, formed another closely related group. One of Holttum's most important contributions was the recognition that the three phegopteroid genera formed a related group (Holttum, 1969, 1971).

Although elements of all of these precladistic and largely intuitive trees have some credence, the molecular data provide a unique new hypothesis of relationships. The phegopteroid genera (*Phegopteris*, *Macrothelypteris*, and *Pseudophegopteris*) appear to be the sister group to all other taxa in the family (Figs. 1, 2). Within the phegopteroid clade, *Macrothelypteris* appears as the sister group to *Pseudophegopteris* and the two *Phegopteris* species. All nodes within the phegopteroid clade have high bootstrap support (Fig. 1).

Following the origin of *Thelypteris s.s.*, which is shown to be basal in the thelypteroid–cyclosoroid clade, the free-veined thelypteroids arise, with greater or lesser bootstrap support for various nodes, and no significant support at all for some of the nodes at the crown of one of the main branches (Fig. 1). *Thelypteris s.s.* is shown to be only distantly related to *Cyclosorus s.s.*, thus refuting the close relationship suggested by Holttum (1982:386).

The cyclosoroids, from *Steiropteris* on up, form a large, mostly unresolved or poorly resolved clade toward the tip of the tree (Figs. 1, 2). This topology supports the contention of Pichi Sermolli that, in general, those elements in the family with anastomosing veins and $x=36$ are evolutionarily derived in the family. In the tree shown, *Dictyocline* and *Leptogramma* appear as sister genera, a result that supports all recent classifications of the family. Most authors, including Iwatsuki (1963), Holttum (1982), and Smith (1990) unite these two segregates in the same genus or subgenus.

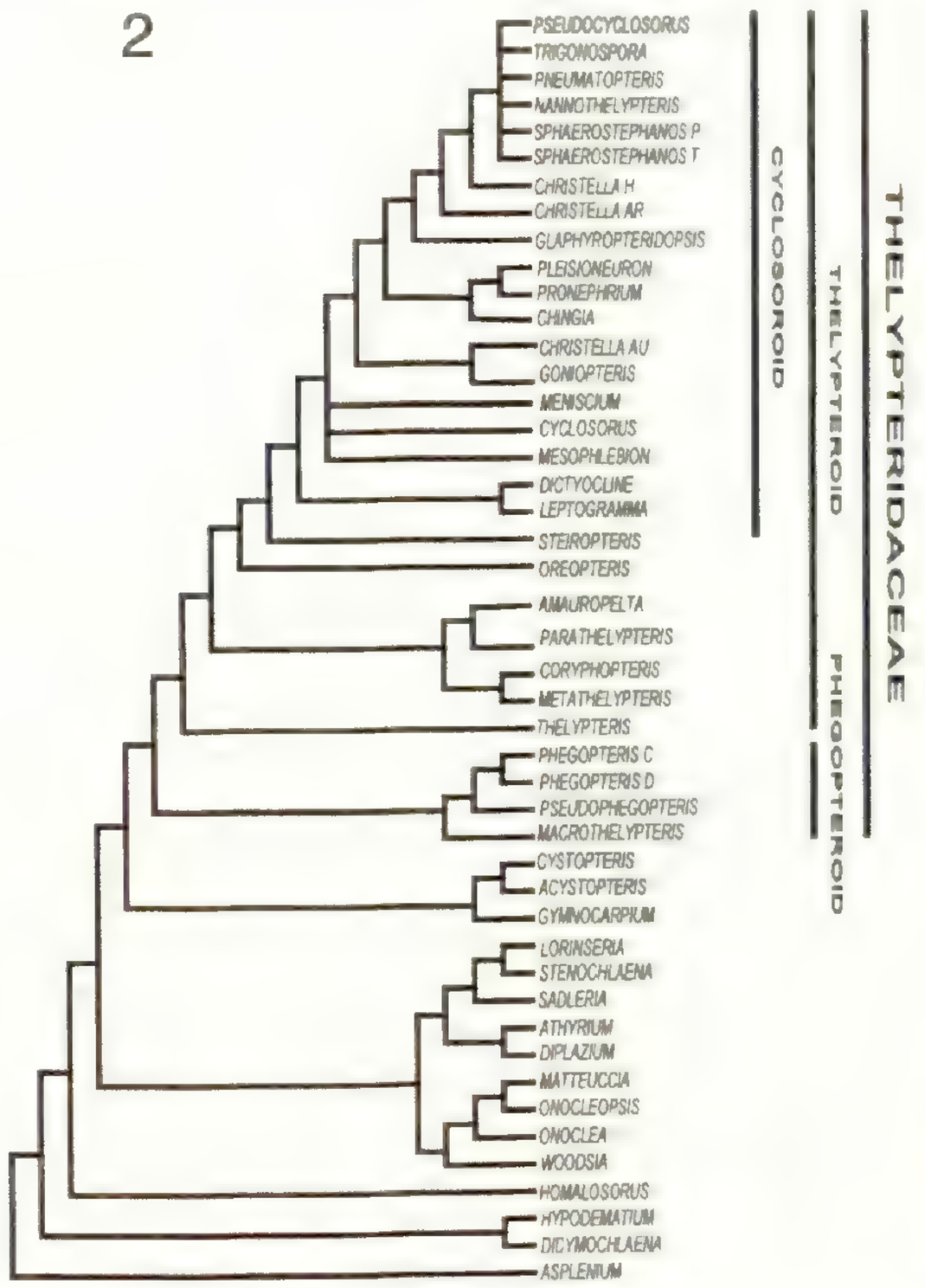
The lack of resolution of various genera within the cyclosoroid clade (Figs. 1, 2) may be an artifact of inadequate sampling or an indication of insufficient variation in the genes sampled; more likely, it also reflects weak distinctions between and among genera within this clade. Several hybrids are known between species in different cyclosoroid genera (Viane, 1985; Quansah & Edwards, 1986; Sledge, 1981; J. Game, unpubl. data). Many of the cyclosoroid generic segregates seem little more than one- or few-character genera, often containing exceptional species that do not fully conform in their diagnostic features (Smith, 1990). We note in this regard that the three species of *Christella* sampled in our study do not form a monophyletic group, and in fact the single New World species sampled, *Christella augescens* (Link) Pic. Serm., is well separated from the other two species. This African and Neotropical group of 23 species (Smith, 1971; Smith, 1990) has sometimes been treated in a different subgenus, subg. *Pelazoneuron*, in contrast to the largely Asian subg. *Christella*.

CHARACTER EVOLUTION.—Below we discuss briefly several characters often applied in circumscription of groups (genera, subgenera, sections) of Thelypteridaceae, expecially those characters that appear to correlate to a significant extent with the results presented here.

Venation.—All students of the family have recognized the importance of venation in the classification and subdivision of the thelypteroid ferns and have utilized that character, whether free, connivent at the sinus, or anastomosing in various ways, as a primary key character and feature delimiting genera or infrageneric taxa within the family (*e.g.*, Christensen, 1907, 1913, 1920; Holttum, 1971, 1982; Tryon & Tryon, 1982:432–453; Smith, 1990). Our results show that the basalmost clades in the family, from the phegopteroid genera through *Amauropelta* and *Parathelypteris* (Fig. 3) are all free-veined or at least have veins that run to the sinus (but do not anastomose). In contrast, most of the groups belonging to the cyclosoroid clade (Fig. 3, shaded boxes) have veins that are either connivent at the sinus, or anastomose at an acute angle below the sinus, or unite at an oblique angle below the sinus with an excurrent vein to the sinus. In some groups, there are multiple series of anastomoses between the costa and pinna margin, as in the neotropical groups *Meniscium* and *Goniopteris*, or in the paleotropical groups *Pronephrium*, *Sphaerostephanos*, and *Dictyocline*. Vein fusion is known to have arisen independently in many leptosporangiate fern lineages, and no doubt has also evolved many times within Thelypteridaceae. Smith (1990:271) pointed out that half of the 20 subgenera of *Cyclosorus s.l.* have both free-veined and anastomosing-veined members. The evolutionary trend toward increased vein anastomosing, however, is unmistakable, and lends support to the overall topology of the tree. Many of the outgroups we used in this study are also free-veined, but certain ones, such as *Onoclea* and *Lorinseria*, also have evolved anastomosing venation.

Anastomosing or reticulate venation in Thelypteridaceae is generally of a simple or very repetitive nature: at most there are simple (unforked), excurrent free veins within areoles, as in *Meniscium*. This pattern of vein reticulation, termed "intersegmental" by Wagner (1979), is so common in many thelypteroid groups that Wagner termed the venation pattern "thelypteroid"; if the pattern is repeated several times, Wagner (and others) have termed it "meniscioid" (after the genus *Meniscium*) or "goniophlebioid" (similar to a venation pattern in the genus *Goniophlebium*, a member of the Polypodiaceae). The various venation types in the family have been discussed and illustrated by Iwatsuki (1962), who described the vein-fusion process from an evolutionary perspective. The regularity of the pattern in thelypteroid ferns contrasts with venation types in many Polypodiaceae and some members of Dryopteridaceae (both with many epiphytic members), which are often much more complex, irregular, and with recurrent (as well as excurrent) and also forked veinlets in the areoles.

Adaxial sulcation.—Holttum (1960) was one of the first to discuss in depth the importance of sulcation, or adaxial grooving, as applied to the systematics of ferns. He soon recognized that all three phegopteroid genera, as well



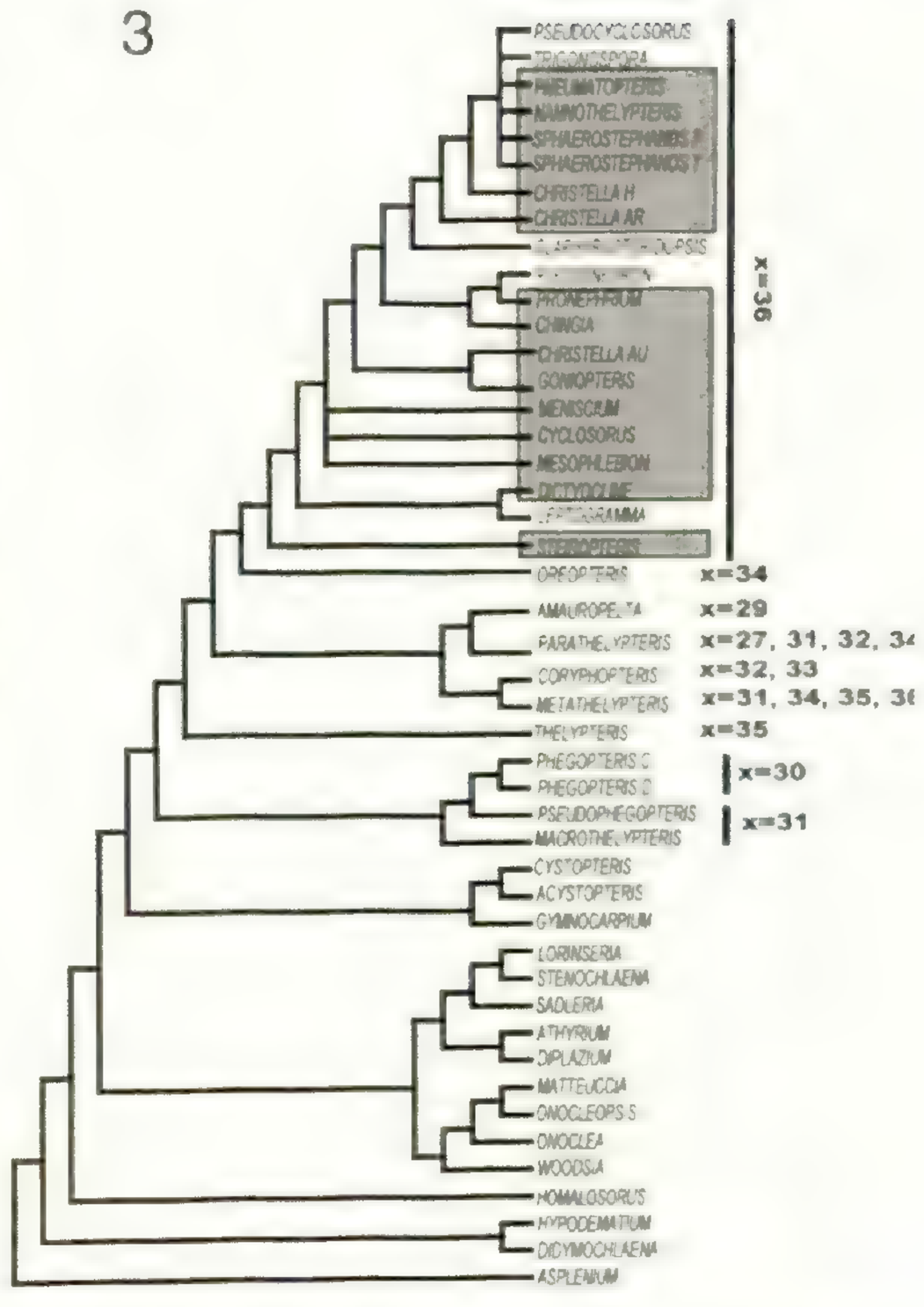


FIG. 2. Strict concensus tree of the 28 most parsimonious trees found in the combined parsimony analysis, based on the chloroplast genes *rps4*, *trnS* spacer, and *trnL* spacer. The tree is rooted on *Asplenium*, and *Asplenium* through *Cystopteris* represent outgroups used in the analysis. Familial and subfamilial groups are indicated at the right. See Table 1 for full names of species and additional voucher data.

FIG. 3. Strict concensus tree of the 28 most parsimonious trees found in the combined parsimony analysis, based on the chloroplast genes *rps4*, *trnS* spacer, and *trnL* spacer. Thelypteroid taxa having connivent or anastomosing veins are indicated by shaded boxes; chromosome base numbers for various groups are shown along the right. See Table 1 for full names of species and additional voucher data.

as *Metathelypteris*, lacked adaxial grooves along the costae (Holttum, 1969, 1982). This contrasts with all other thelypteroid ferns, which have the costae adaxially sulcate. As Holttum (1960) noted, some genera of higher leptosporangiate ferns (*e.g.*, *Dryopteris* and *Polystichum*) have the grooves of one axis continuous with the grooves of the axes of greater (and often lesser) orders, but continuous grooving is unknown in Thelypteridaceae.

The grooving of many of the outgroups used in this study has not been sufficiently studied, but most appear to be adaxially grooved, including both *Cystopteris* and *Gymnocarpium*, the apparent closest relatives.

Blade dissection.—With rare exceptions, blades of all cyclosoroid and thelypteroid genera in Figure 2 are pinnate-pinnatifid or less divided. Those few species that have more divided fronds (*e.g.*, *Thelypteris* (*Amauropelta*) *pteroidea* (Klotzsch) R. M. Tryon) are clearly derived, and barely 2-pinnate. A few members of some cyclosoroid clades have simple or merely pinnatifid

blades, *e.g.*, certain species of *Meniscium*, *Goniopteris*, *Pronephrium*, and *Sphaerostephanos*. In contrast, the phegopteroid genera have blades that are bipinnate-pinnatifid (or even more divided), or the blades are bipinnatifid or tripinnatifid (*Phegopteris*).

Dimorphism.—Frond (blade) dimorphism is nowhere strongly pronounced in the family, as it is in many other fern families, but does occur on a subtle to moderate scale (subdimorphism) in many (but not all) members of the cyclosoroid clade, *e.g.*, *Christella*, *Goniopteris*, *Meniscium*, *Pronephrium*, and *Sphaerostephanos*. Dimorphism is nearly totally lacking among members of the phegopteroid clade, as well as most members of the thelypteroid clade; exceptions to this are found in the subdimorphic group *Thelypteris s.s.*, and subtly in *Coryphopteris* and *Parathelypteris*. There appears to be a positive correlation between degree of vein anastomosing and blade dimorphy in the family, *i.e.*, those groups that have significant vein reticulation also are more likely to be subdimorphic. Hemidimorphism (fertile-sterile differentiation on separate parts of the same leaf; Wagner, 1977) is essentially unknown in Thelypteridaceae. As pointed out by Wagner (1979), dimorphy appears to be a character that is valuable mainly at the species level, not at generic rank, and the evolution of dimorphy (or subdimorphy) has occurred independently many times in the ferns and in the Thelypteridaceae. It is doubtful that this character is of any help in delimiting clades within this family.

Among outgroups used in this study, most have monomorphic fronds, but members of the Blechnaceae (*e.g.*, *Lorinseria* and *Stenochlaena*) are often, but not always (*e.g.*, *Doodia*, *Sadleria*, a few *Blechnum* species), strongly dimorphic, as are members of the onocleoid ferns (*Onoclea* and *Matteuccia* in our sample).

Spore morphology.—Spores of thelypteroid ferns are generally monolete and kidney-bean-shaped, with a relatively thick, folded, cristate, reticulate, or echinate perispore. Spore surveys of thelypteroid genera suggest that spore ornamentation is, in some cases, correlated with the segregate taxonomy (Wood, 1973; Tryon & Tryon, 1982; Tryon & Lugardon, 1991). One of the best characterized segregates appears to be *Amauropelta*, with a uniformly reticulate perine. *Pseudophegopteris* and *Macrothelypteris* have similar spore ornamentation, but the reticulate network is coarser and of lower relief than in *Amauropelta*. Species of *Stegnogramma*, in the cyclosoroid alliance, have distinctive echinate spores, but these are not unlike many species of *Sphaerostephanos* and some other Old World cyclosoroid segregates, particularly *Pneumatopteris* (Tryon & Lugardon, 1991:407). In general, members of the cyclosoroid alliance have more coarsely folded or echinate ornamentation than do members of the phegopteroid and thelypteroid alliances. Spores of the neotropical *Steiropteris* are very broadly and coarsely winged, with a fine, low reticulate network between wings (Smith, 1980); similar spores, but lacking the low reticulum, are also found also among New World *Goniopteris* and *Meniscium*, as well as in the paleotropical group *Mesophlebion* (Tryon & Lugardon, 1991:401), and these similarities (as well as others) suggest a possible close relationship. Spores of *Trigonospora* are trilete, the only

such spores in the family; this is unquestionably a derived condition in Thelypteridaceae, and not likely an indication of relationship to the Jurassic fossil *Aspidistes*, as suggested by Holttum (1982) and others. No doubt, there is much more evidence of a taxonomic nature to be gleaned from a thorough and comparative study of spore morphologies in the family.

Biogeography.—Most of the segregate genera recognized in thelypteroid ferns are restricted either to the New World or to the Old World. In the tropics, exceptions are *Cyclosorus s.s.*, *Christella* (predominantly Old World) and *Amauropelta* (predominantly New World). In north-temperate areas, *Oreopteris*, *Thelypteris*, and *Parathelypteris* are amphi-oceanic, occurring in both North America and Eurasia.

The cyclosoroid clade (Fig. 2) is entirely tropical or subtropical in its current distribution; few members extend above 25° north latitude. Contrastingly, of the basal thelypteroid segregates, *Thelypteris s.s.* (except the segregate *T. confluens* (Thunb.) C. V. Morton also occurs in south temperate regions), *Oreopteris*, and *Parathelypteris* are mostly found north of 25° north latitude, and *Metathelypteris* also has many temperate representatives. *Amauropelta* and *Coryphopteris* are the only largely tropical segregates. In the phegopteroid alliance, *Phegopteris* itself is north-temperate in distribution, in both eastern Asia and North America, while *Macrothelypteris* and *Pseudophegopteris* are restricted essentially to the Old World tropics and subtropics (*M. torresiana* (Gaudich.) Ching is widely naturalized in the New World). Several outgroups sampled are cosmopolitan (*e.g.*, *Asplenium*, *Cystopteris*, *Woodsia*); others are more restricted (*e.g.*, *Lorinseria* and *Sadleria*).

We note that the phegopteroid clade and also the basal clades of the thelypteroid lineage are, for the most part, Laurasian in distribution. This distribution perhaps indicates an east Asian origin, north of the Tethys sea, for the family. Although both *Pseudophegopteris* and *Macrothelypteris* are confined to the Old World (in their native distribution), the other phegopteroid and most of the basal thelypteroid genera are found in both the Old World and New World, and most are decidedly northern in their general distribution. Only higher in the thelypteroid lineage, in particular the cyclosoroid groups, are the genera confined to either the Neotropics or Paleotropics, or may be Gondwanan in origin. Fossil evidence for the Thelypteridaceae is nearly confined to the Cenozoic and is nomenclaturally confused because of the historic inclusion of thelypteroid ferns in the distantly related dryopteroid assemblage. The best known and earliest examples of thelypteroid fossils appear to be from Eocene strata of North America and Europe (Collinson, 2001:209–212). Further study and re-evaluation of identifications in light of modern systematic knowledge of extant members is needed to determine whether the fossil evidence adds support to a Laurasian origin for the family.

Chromosome numbers.—Most of the segregate genera of Thelypteridaceae are characterized by having a single base chromosome number. All representatives of all of the cyclosoroid genera sampled (Fig. 3) are known to be based on $x = 36$, and many species in most of these segregates have been sampled.

Chromosome base numbers are known for all groups except *Nannothelypteris*, which has been combined with *Pronephrium* sect. *Dimorphopteris* by Smith (1990) as suggested by Holttum (1982:538). Of the phegopteroid genera (Fig. 3), *Phegopteris* is consistently $x = 30$, with all three species counted; *Pseudophegopteris* is $x = 31$, with eight species counted; and *Macrothelypteris* is also $x = 31$, with four species counted. The situation in the free-veined thelypteroid segregates is more complex. There appears to be a dysploid series of numbers that characterizes the various groups (Fig. 3; Lovis 1977). *Thelypteris s.s.* is consistently $x = 35$; Oreopteris is $x = 34$ (all three species counted); *Coryphopteris* species are $x = 32$ and 33 (Smith, unpubl.), but with few counts available; reports for *Metathelypteris* are mostly $x = 31$, 34, 35, and 36 (five species counted); *Parathelypteris* is variously $x = 27$, 31, 32, 34, with the first two numbers being most commonly reported; and *Amauropelta* is consistently $x = 29$, with ca. 25 species counted. Whether the multiple numbers for some of the segregates indicate unnaturalness in the existing classifications or accurately reflect chromosomal variation among (and even within) closely related species remains to be determined. It does appear, however, that there is some chromosomal instability within the basal thelypteroid group of genera.

Summary and Conclusions

Although taxon sampling is still only preliminary, we conclude that the family Thelypteridaceae is monophyletic, with a high degree of certainty. It excludes a few genera that have sometimes been included in the family by Ching (*e.g.*, *Hypodematium*; Ching, 1963; *Trichoneuron*; Ching, 1978) but exactly coincides with the circumscription given by Iwatsuki (1964), Holttum (1971), Pichi Sermolli (1977), and Smith (1990). Although our sampling is not yet sufficient to favor one of the many classifications (Morton, 1963; Iwatsuki, 1964; Holttum, 1971, 1982; Ching, 1963, 1978; Pichi Sermolli, 1977; Smith (1990) of the family over another, our analysis suggests that recognition of an intermediate number of genera may be a reasonable taxonomic course. The three phegopteroid genera appear to be basal in the family and clearly sister to the remaining genera and subgenera.

Acknowledgments

This projects builds on work done by several giants in the field of pteridology: Carl Christensen, who provided keen insight and the first monographs of subgroups of neotropical thelypteroid ferns in 1907 and 1913; Ren Chang Ching, who characterized several of the subgroups in Asia; Conrad Morton, who expanded our knowledge of *Thelypteris* in the Neotropics in the 1940s through the 1960s; and Richard Eric Holttum, who continued the monographic tradition with revisions of most of the paleotropical subgroups in the family in the 1970s and 1980s. Without their initial insights, there would be no firm basis for attempting a study of the phylogenetic relationships in the family. In addition, Smith acknowledges the friendship and early assistance of Herb Wagner, who provided material of *Cyclosorus*, material which was to serve as the foundation of a Ph.D. thesis and provide impetus for a lifelong interest in Thelypteridaceae.

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Two New Species of Moonworts (*Botrychium* subg. *Botrychium*) from Alaska

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ABSTRACT.—*Botrychium tunux* and *Botrychium yaaxudakeit*, new species of moonworts currently known only from southern Alaska, are described and illustrated. These ferns are distinguished from *B. lunaria*, with which they have been confused, by allozyme data and their morphological characteristics. Ploidy levels of *B. tunux* (diploid) and *B. yaaxudakeit* (tetraploid) are inferred from allozyme patterns. A key to Alaskan moonworts is presented.

Before 1995, little attention was paid to Alaskan moonworts, *Botrychium* subg. *Botrychium*. Information gained from rare plant surveys since then has improved our knowledge of moonwort abundance, distribution, and relationships. Seven species of moonworts are now known in southern Alaska: *Botrychium ascendens* W.H. Wagner, *B. lanceolatum* (S.G. Gmel.) Ångström, *B. lunaria* (L.) Sw., *B. minganense* Vict., *B. pinnatum* H. St. John, the new species *B. alaskense* W.H. Wagner & J. R. Grant, and two additional new species described here, *B. tunux* and *B. yaaxudakeit*.

During a 1980 USDA Forest Service rare plant survey, several *Botrychium* identified as *B. lunaria* (*M. C. Muller 3806*, ALA, TNFS) were collected in sandy upper beach meadows near Yakutat, in southeastern Alaska. In 1986, W. H. Wagner and F. S. Wagner (1986) described a new moonwort, *Botrychium ascendens*, and re-identified some of the 1980 specimens as *B. ascendens*; other specimens on the herbarium sheets remained *B. lunaria*. The inclusion of Alaska in the range of *B. ascendens* in Volume 2 of the Flora of North America (W. H. Wagner, 1993) was based on the 1980 Muller collection.

In July 1995, the Forest Service conducted rare plant surveys to re-locate the Yakutat *B. ascendens*. Specimens were collected at the 1980 site and elsewhere in the Yakutat area. These were sent to W. H. Wagner for identification. He determined that in 1995, only *B. minganense* was collected at the 1980 *B. ascendens* site, but felt that some of the other specimens collected in the Yakutat area might be an undescribed species. In a study of the Yakutat Botrychiums in 1996, David Wagner recognized two forms of *B. lunaria* and recommended further study. In 1997, the Forest Service sent assorted specimens of tentatively identified *B. lunaria* and other *Botrychium* specimens to D. R. Farrar, to be analyzed through starch gel enzyme electrophoresis.

Farrar's analysis identified *B. minganense*, *B. lunaria*, and two moonworts previously unknown to science. These new moonworts, a diploid and an allotetraploid, resemble *B. lunaria* most closely in morphology, but are distinct genetically and morphologically from all other known moonworts. To our knowledge the diploid has not been previously collected. Previous collections of the tetraploid had been included in *B. lunaria*.

Genetic comparison of *B. tunux* with *B. lunaria* at 18 allozyme loci yielded a value for Nei's (1978) genetic identity (GI) of 0.5102 (Farrar, unpubl. data). This represents a level of genetic similarity only slightly greater than that between *B. lunaria* and *B. simplex* (GI = 0.4646) and is significantly less than the similarity between *B. lunaria* and its sister taxon, *B. crenulatum* (GI = 0.7015). Absence of fixed heterozygosity at any enzyme locus implies that plants of *B. tunux* are diploid. Presence of fixed heterozygosity and a spore size significantly larger than that of *B. lunaria* implies that plants of *B. yaaxudakeit* are tetraploid. Allozyme patterns also show that one ancestral diploid parent of *B. yaaxudakeit* is North American *B. lunaria*; the other ancestral diploid is probably not among the known North American diploid species. Preliminary investigation of European *B. lunaria* indicates that *B. yaaxudakeit* may be the result of ancient hybridization between genetically distinct North American and Eurasian genotypes of *B. lunaria*.

Key to the Species of *Botrychium* Subg. *Botrychium* of Southern Alaska

1. Trophophores (photosynthetic portions of the blade) bipinnate or pinnate-pinnatifid . . . . . 2.
1. Trophophores pinnate . . . . . 4.
2(1). Trophophores triangular, with three nearly equal axes; pinnae narrowly lanceolate . . . . . *B. lanceolatum*
2. Trophophores triangular to ovate, with one principal axis; pinnae broadly lanceolate to ovate . . . . . 3.
3(2). Pinna bases cuneate; pinna apices acute . . . . . *B. alaskense*
3. Pinna bases obtuse to truncate; pinna apices rounded . . . . . *B. pinnatum*
4(1). Basal pinnae narrowly fan-shaped to oblong with the blade spanning an arc less than 90°; pinnae remote . . . . . 5.
4. Basal pinnae broadly fan-shaped with the blade spanning an arc greater than 90°; pinnae approximate to overlapping . . . . . 6.
5(4). Trophophores sessile; pinnae strongly ascending, their margins dentate to lacerate; sporangia usually few on the proximal trophophore pinnae . . . . . *B. ascendens*
5. Trophophores stalked; pinnae spreading to ascending, oblong to narrowly fan-shaped, their margins entire to undulate; sporangia usually lacking on the trophophore pinnae . . . . . *B. minganense*
6(4). Basal pinnae asymmetrical with the basal portion expanded; mature sporophore stalks shorter than or equal to the trophophores; basal sporophore branches spreading and twisted; plants 6–12 cm tall . . . . . *B. tunux*
6. Basal pinnae generally symmetrical; mature sporophore stalks longer than the trophophores; basal sporophore branches ascending and straight; plants 8–25 cm tall . . . . . 7.
7(6). Pinnae strongly overlapping one another and the rachis; basal pinnae spanning an arc greater than 180°; basiscopic inner margins of the basal pinnae strongly recurved; spores 45 (43–48) μm in longest diameter . . . . . *B. yaaxudakeit*

7. Pinnae approximate to somewhat overlapping, not overlapping the rachis; basal pinnae spanning an arc of less than 180°; basiscopic inner margins of the basal pinnae linear to moderately concave; spores 36 (34–39) μm in longest diameter . . . . . *B. lunaria*

Botrychium tunux Stensvold & Farrar, *sp. nov.*—TYPE: U.S.A.: Alaska: Yakutat area, ca. 5 km W of Yakutat, on the Phipps Peninsula ca. 1.5 km SE of Point Carrew, just N of the mouth of the Ankau River, ca. 1.5 m elev., 25 Jul 2001, *M. C. Stensvold & D. R. Farrar 7936* (holotype ISC; isotypes ALA, NY, TNFS, US, WTU). **Figs. 1c, 2c, 3b, 3c.**

Plantae supraterraneae 6–12 (ave. 9) cm altae; stipites communes 0–3 (ave. 1.5) cm longi. Trophophora flavovirentes coriaceae oblongae. Paria pinnarum plus minusve perpendicularia ad rhachim, separata vel leviter imbricata; pinnae basales 7–20 (ave. 13) mm longae, 7–18 (ave. 12) mm latae, sessiles, flabellatae, angulo ad basim 120–180°, saepe asymmetricae, parte basali expanso, margine externa integra vel interdum lobata, sinibus rotundatis. Stipites sporophora 2.5–5 (ave. 4) cm longi, trophophoria aequantes vel breviores. Sporae 38–42 μm diametro.

Rhizomes erect, unbranched, their apex 2–5 cm below the soil surface, bearing 6–22 (ave. 14) fleshy roots; gemmae absent. Above-ground plants 6–12 (ave. 9) cm tall with a common stalk 0–3 (ave. 1.5) cm long. Trophophores yellow-green, leathery; stalks 0–1 cm long; blades 2.5–7 (ave. 4) cm long, 2–4 (ave. 3) cm wide, narrowly ovate to ovate, once pinnate. Pinna pairs 4–6, more or less perpendicular to the rachis, separated to slightly overlapping, not overlapping the rachis. Basal pinnae 7–20 (ave. 13) mm long, 7–18 (ave. 12) mm wide, sessile, fan-shaped spanning an arc of 120–180°, often asymmetrical, with the basal portion expanded; basiscopic inner margins straight to shallowly concave; outer pinna margins entire or occasionally incised or lobed with rounded sinuses; veins dichotomous, 3–5 major veins entering the pinna base, 45–55 veins ending at the margins. Sporophores 5–10 (ave. 7) cm long; sporophore stalks 2.5–5 (ave. 4) cm long and shorter than or equaling the length of the trophophore; sporangia-bearing portion erect, 1–2-pinnate, broadly ovate in outline, branches 4–6, ascending to spreading, especially the lowermost, which are often twisted such that the sporangia project downward; sporangia partially embedded in the distally thickened sporangiophore branches. Spores 38–42 (ave. 40) μm in longest diameter, released later than those of *B. lunaria*. Apparently diploid.

This species is morphologically most similar to *B. lunaria*, from which it can be distinguished by its short stature, short common stalk, frequently stalked, ovate trophophore, asymmetrical pinnae with their basiscopic side expanded, entire margins commonly cleft by shallow incisions with rounded sinuses, and sporophore stalks seldom exceeding the height of the trophophore (Figs. 1c, 2c). *Botrychium lunaria* is taller in stature (to 12 cm), has a longer common stalk (to 3.5 cm), and has an oblong to narrowly elliptic, generally short-stalked or unstalked trophophore. Its fan-shaped pinnae are

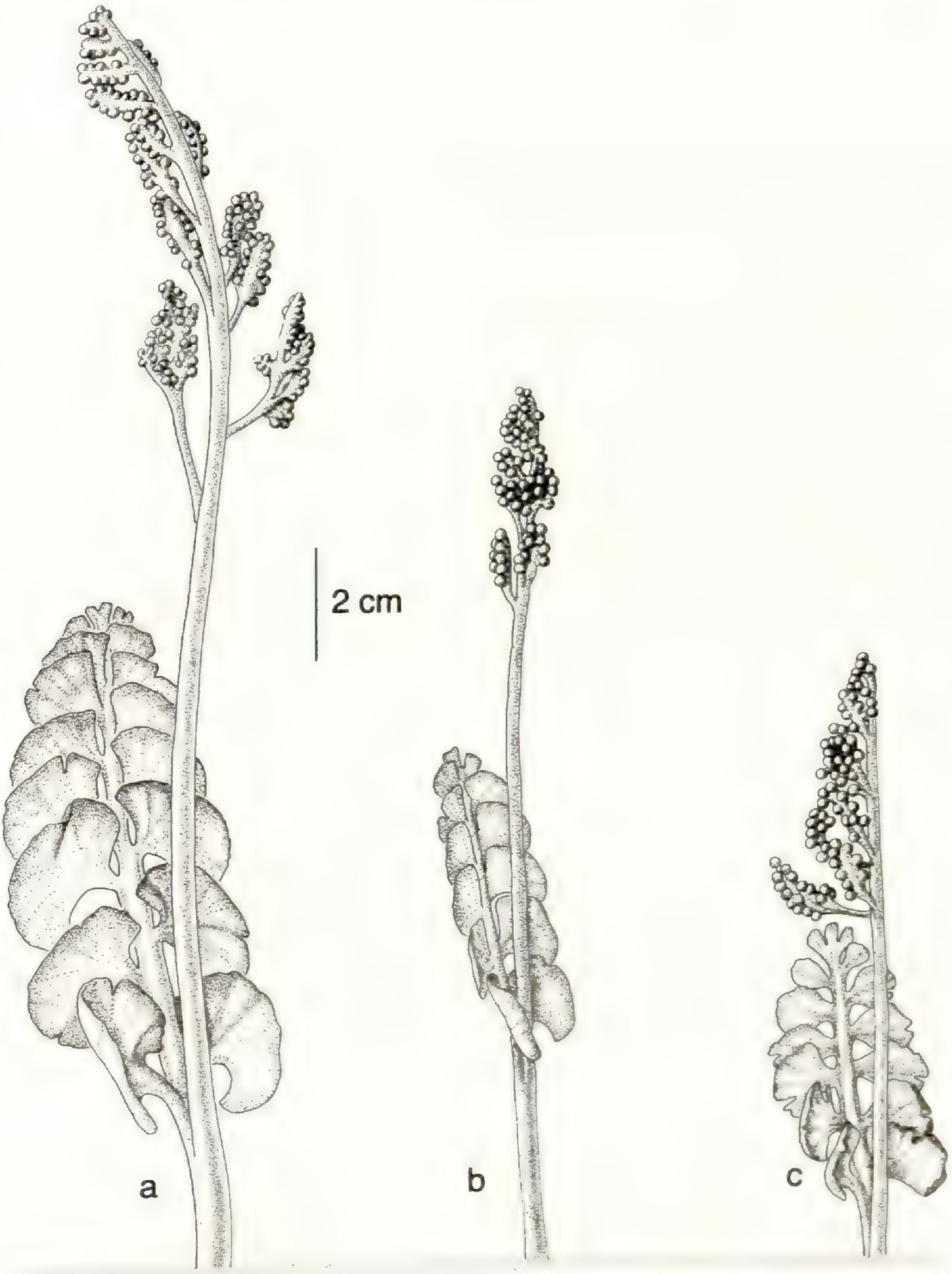

FIG. 1. Illustrations of three species of *Botrychium* showing the differences in stature and morphology. Fig. 1a. *B. yaaxudakeit*. Fig. 1b. *B. lunaria*. Fig. 1c. *Botrychium tunux*.

generally symmetrical, or if they are asymmetrical, the acroscopic side is larger, and if cleft, the clefts have acute sinuses, and the sporophore stalks at maturity usually exceed the height of the trophophore (Figs. 1b, 2b).

PARATYPES.—U.S.A.: Alaska: Yakutat area, E shore of the Phipps Peninsula ca. 1.5 km SE of Point Carrew, just N of the mouth of the Ankau River, *Stensvold 6854,7280* (TNFS), *Farrar 99-07-23-2* (ISC); Blacksand Spit, off the mouth of the Situk River, *Stensvold 7949* (ISC), *Farrar 01-07-27-1* (ISC); Yakutat forelands, ca. 68 km SE of Yakutat, on NW–SE-oriented sandspit separating the Pacific Ocean from the mouth of the Akwe River, ca. 3 km W of the junction of the Akwe and Ustay Rivers, *V. Harke s.n.* (TNFS), *Stensvold 7305, 7770* (both TNFS), *Farrar 99-07-24-2, 99-07-24-6* (both ISC); Yakutat forelands, ca. 80 km SE of Yakutat, Dry Bay, NW side of the mouth of the Alsek River, *Farrar 99-07-25-2* (ISC).

Botrychium tunux is known from four locations in the Yakutat area: Point Carrew near the village of Yakutat, Blacksand Spit off the mouth of the Situk River, Akwe beach, and the northwest shore of Dry Bay, all on the Yakutat forelands, an area 80 km long extending between Pt. Carrew and Dry Bay in habitats adjacent to saltwater but not influenced by saltwater inundation. Searches for *B. tunux* in similar habitat on the Copper River Delta barrier islands, some of the Malaspina Glacier forelands, Cross Sound, Icy Strait, Glacier Bay and Lynn Canal areas were unproductive, although other *Botrychium* species were found. We believe potential habitat for both of the new species extends along the Alaska coastline for 250 km in either direction from Yakutat. Because the area is frequented by storms and accessible only by aircraft capable of beach landings, this coastline is botanically underexplored. A single collection of six plants from the Nutzotin Mountains ca. 13 km northwest of Chisana (ca. 305 km. NNW of Yakutat) differ somewhat genetically and morphologically from the coastal *B. tunux*, but may be a conspecific population long isolated from coastal plants.

In the Yakutat area, *B. tunux* grows in habitats ranging from open sand on dunes and upper beaches to well drained upper beach meadows with sandy substrates (Figs. 3b–d). The plants may occur as scattered individuals or in loosely associated groups. Vascular plants most commonly associated with *B. tunux* include *Achillea borealis*, *Festuca rubra*, *Fragaria chiloensis*, *Gentianella amarella*, *Leymus mollis*, *Lupinus nootkatensis*, *Oxytropis campestris*, and *Rhinanthus crista-galli*. Bryophytes commonly found with *B. tunux* include *Ceratodon purpureus*, *Racomitrium canescens*, and *Rhytidiadelphus squarrosus*. The moonworts *B. ascendens*, *B. lunaria*, *B. minganense*, and *B. yaaxudakeit* also grow in these meadows. Vascular plant and bryophyte cover can range from a trace to 100%, with the percent cover and species number increasing landward. These vegetation assemblages are early seral stages (Fig. 3d). Beaches in the Yakutat area are formed by longshore sand deposition or isostatic rebound and tectonic uplift. *Picea sitchensis* and *Alnus viridis* colonize the meadows and develop into *Picea sitchensis* forests

FIG. 2. Pressed specimens of *Botrychium* clipped just above ground level, from the type locality of the new species near Yakutat, Alaska. Fig. 2a. *Botrychium yaaxudakeit*. Fig. 2b. *B. lunaria*. Fig. 2c. *B. tunux*.

FIG. 3. *Botrychium yaaxudakeit* and *Botrychium tunux* at the type locality at Ankau Beach near Yakutat, Alaska. Fig. 3a. Two plants of *B. yaaxudakeit*. Fig. 3b. A single plant of *B. tunux*. Fig. 3c. *B. tunux* (left) and *B. yaaxudakeit* (right) showing the typical stature of the two species. Fig. 3d. Sparsely vegetated upper beach sand habitat of *B. yaaxudakeit* and *B. tunux*, with a Sitka alder (*Alnus viridis* subsp. *sinuata*) beach-forest ecotone and a young Sitka spruce (*Picea sitchensis*) forest in the background.

(Fig. 3d), while new meadows develop on recently formed land to the seaward (Shephard, 1995).

Related plants in the Nutzotin Mountains were widely scattered on a sparsely vegetated southwest-facing slope in the Gold Hill vicinity at an elevation of about 1615 m on alpine scree slopes. Vascular plants at this location include *Erigeron caespitosus*, *Senecio cymbalaria*, *Arnica frigidia*, *Silene uralensis* subsp. *uralensis*, *Anemone drummondii* var. *lithophila*, and *Elymus trachycaulus* subsp. *violaceus*. The moonworts *B. ascendens*

and *B. minganense* also grow on this scree slope. Further study of plants from this and similar inland sites is needed.

In reviewing herbarium material and literature, we found no prior collections for *B. tunux*. Neither Coville (1895) nor Stair and Pennell (1946) mention any species of *Botrychium* in their papers describing the flora of the Yakutat area. According to their reports, they likely visited the type locality. During the 1899 Harriman Alaska Expedition, Coville and Kearney collected *B. lunaria* in Yakutat Bay (Merriman, 1910), and in 1904 C. V. Piper collected what appears to be *B. yaaxudakeit* at the type locality. We suspect that *B. tunux* may not have been collected because of its dwarfed and malformed appearance relative to the larger *B. lunaria* and *B. yaaxudakeit*, with which it grows.

The epithet *tunux* was chosen by the elders of the Yakutat Tlingit people and honors Tunux, the Tlingit warrior who initiated the 1805 attack on the Russian fort located near the type locality. As a result of the battle, the Russians were removed from Yakutat and never returned. Tunux was in the Eagle Moiety and the Teikweidí (Brown Bear) Clan. The word Tunux is pronounced with a guttural "x" similar to the German "ach," making the name sound like "toonook," with a soft "k."

Botrychium yaaxudakeit Stensvold & Farrar, *sp. nov.*—TYPE: U.S.A.: Alaska: Yakutat area, ca. 5 km W of Yakutat, on the Phipps Peninsula ca. 1.5 km SE of Point Carrew, just N of the mouth of the Ankau River, ca. 1.5 m elev., 25 Jul 2001, *M. C. Stensvold & D. R. Farrar 7937* (holotype ISC; isotypes ALA, NY, TNFS, UC, US, WTU). **Figs. 1a, 2a, 3a, 3c.**

Plantae supraterraneae 8–25 (ave. 18) cm altae; stipites communes 1–5 (ave. 2.5) cm longi. Trophophora viride nitidae coriaceae ovatae. Paria pinnarum plus minusve perpendicularia ad rhachim, valde imbricata; pinnae basales 7–30 (ave. 18) mm longae, 9–32 (ave. 20) mm latae, breviter petiolatae, late flabellatae, angulo ad basim 180–250°, plerumque symmetricae; margine interna basiscopica valde recurvata; margine externa integra vel undulata vel interdum denticulata, interdum fissurata sinibus angulatis. Stipites sporophorora 5–9 (ave. 7) cm longi, excedentes trophophoria. Sporae 43–49 μm diametro.

Rhizomes erect, unbranched, their apex 2–4 (ave. 5) cm below the soil surface, bearing 5–24 (ave. 13) fleshy roots; gemmae absent. Above-ground plants 8–25 (ave. 18) cm tall, with a common stalk 1–5 (ave. 2.5) cm long. Trophophores green, leathery; stalks 0–0.5 cm long; blades 1.75–11 (ave. 7) cm long, 1.25–6 (ave. 4) cm wide at the base, oblong to ovate, once pinnate. Pinna pairs 4–7, angled toward the apex, strongly overlapping one another, the anterior portion overlapping the rachis. Basal pinnae 7–30 (ave. 18) mm long, 9–32 (ave. 20) mm wide, short-stalked, fan-shaped, spanning an arc of of 180° to 250°, usually symmetrical; basiscopic inner margin strongly recurved; outer pinna margins entire to undulate, occasionally denticulate or

occasionally shallowly cleft with angular sinuses; veins dichotomous, 3–5 major veins entering the pinna base, 90–120 veins ending at the margins. Sporophores 8–18 (ave. 14) cm long; sporophore stalks 5–9 (ave. 7) cm long and longer at maturity than the length of the trophophore; sporangia-bearing portion erect, 1–2 pinnate, broadly lanceolate to narrowly ovate in outline, the branches 6–8, basal branches ascending and not twisted. Spores 43–48 (ave. 45) μm in longest diameter, released earlier or at about the same time as those of *B. lunaria*. Apparently tetraploid.

This species is morphologically most similar to *B. lunaria*, from which it can be distinguished by its taller stature, shorter common stalk, more ovate trophophore, and strongly overlapping pinnae that also overlap the rachis. Its basal pinnae are often short-stalked with a span of over 180° and have strongly recurved basiscopic inner margins (Figs. 1a, 2a). *Botrychium lunaria* is shorter in stature (12 cm), has a longer common stalk (3.5 cm), oblong to narrowly ovate, usually unstalked trophophores, and pinnae that generally do not strongly overlap one another or the rachis, are sessile with a span of less than 180°, and have slightly concave basiscopic inner margins (Figs. 1b, 2b). The spores of *B. yaaxudakeit* are significantly larger than those of *B. lunaria*.

PARATYPES.—Alaska, Yakutat area, E shore of the Phipps Peninsula ca. 1.5 km SE of Point Carrew, just N of the mouth of the Ankau River, *M. C. Muller 3808, Stensvold 7272,* (TNFS), *M. Shephard 22* (TNFS), *Stensvold 6854-2* (TNFS), *Farrar 99-07-23-3* (ISC); Ankau River, 31 Aug – 1 Sep 1904, *C. V. Piper 4565* (US); Yakutat forelands, ca. 68 km SE of the town of Yakutat, on a NW–SE-oriented sandspit separating the Pacific Ocean from the mouth of the Akwe River, ca. 3 km W of the junction of the Akwe and Ustay Rivers, *Stensvold 7305* and *7773* (both TNFS), *Farrar 99-07-24-7* (ISC); Yakutat forelands ca. 80 km SE of Yakutat, NW side of the mouth of the Alsek River, well drained upper beach meadow, *Farrar 99-07-25-3* (ISC); Glacier Bay, near Rush Point, *Stensvold 7532-1* (ISC); Glacier Bay, N of Gloomy Knob at the outlet of Vivid Lake, *Stensvold 7554* (ISC); Glacier Bay, near the mouth of Tarr Inlet, *Stensvold 7669-1* (ISC); roadside along the Richardson highway 11.5 km NW of the Salcha River crossing, at the junction with Johnson Road, 24 Jun 1999, *W. H. Wagner s.n.* (ISC).

In southeastern Alaska, *B. yaaxudakeit* is known from seven locations. Four of these are the same Yakutat locations where *B. tunux* grows. Searches for *B. yaaxudakeit* in similar habitat on the Copper River Delta barrier islands, some of the Malaspina Glacier forelands, in Cross Sound, Icy Strait, Glacier Bay, and Lynn Canal areas resulted in *B. yaaxudakeit* discoveries at three sites in Glacier Bay. The Yakutat habitats are described in the *B. tunux* section of this paper and pictured in Figure 3. In Glacier Bay, *B. yaaxudakeit* was found in three areas, near Rush Point, north of Gloomy Knob at the outlet of Vivid Lake, and near the mouth of Tarr Inlet, in habitats ranging from gravelly, sandy upper beaches to lush, well drained upper beach meadows with sandy substrates. The plants occur as scattered individuals or in

loosely associated groups. Vascular plants most commonly associated with *B. yaaxudakeit* in Glacier Bay include *Achillea borealis*, *Fragaria chiloensis*, *Festuca rubra*, *Honckenya peploides*, *Lupinus nootkatensis*, *Leymus mollis*, *Gentianella amarella*, *Heracleum lanatum*, *Moehringia lateriflora*, *Oxytropis campestris*, *Rhinanthus crista-galli*, *Rubus stellatus*, and *Taraxacum* spp. Bryophytes commonly found with *B. yaaxudakeit* include *Rhytidiadelphus squarrosus*, *Ceratodon purpureus* and *Racomitrium canescens*. The moonworts *B. ascendens*, *B. lunaria*, and *B. minganense* also grow in these meadows. Vascular plant and bryophyte cover was 100%, except at the Tarr Inlet site where plant cover was 30%. A single plant collected in south-central Alaska near Fairbanks by W. H. Wagner (24 June 1999) was confirmed by isozyme electrophoresis to be *B. yaaxudakeit*. This plant grows in roadside vegetation along the Richardson highway.

In reviewing herbarium material, we found prior collections of *B. yaaxudakeit* that had been identified as *B. lunaria*. C. V. Piper collected what appears to be *B. yaaxudakeit* at the type locality: Ankau River, 31 Aug–1 Sep 1904, *C. V. Piper 4565* (US 421289). Collections made at the type locality in more recent years include: Yakutat ca. 3 mi W of town on the eastern Phipps Peninsula just N of the Ankau River, forest, sandy beach ecotone, 17 Jul 1980, *M. C. Muller 3808* (TNFS) and Yakutat, in the back-beach meadow at Point Carrew, 11 Jul 1993, *Shephard 22* (TNFS). Two specimens from Glacier Bay National Park and Preserve may also be *B. yaaxudakeit*, Glacier Bay, Beardslee Islands, 3 Aug 1921, *J. P. Anderson 1155* (US), and bedrock knob north of Vivid Lake, 10 Jul 1990, *K. Bosworth, s. n.* (Glacier Bay National Park and Preserve Herbarium).

The elders of the Yakutat Tlingit people chose the epithet, *yaaxudakeit*, to honor Yaa Xu da Keit'. He was the leader of an ancient clan living at the base of the mountain now known as Mt. St. Elias, and purchased Yakutat for the Copper River people. Yaa Xu da Keit' was from the Raven Moiety and the Kwaashkikwaan (Humpback Salmon) Clan. Yaa Xu da Keit' is pronounced with a guttural "x" and "k" and a pinched "t," making the name sound like "yaa khoo da kayt".

Acknowledgments

We thank elders of the Yakutat Tlingit people for providing names for the new species. We thank David Wagner for his analysis of the Yakutat *Botrychium*. Vince Harke for his enthusiasm and logistical assistance, Lynn Clark for the Latin descriptions, Anna Gardner for preparing the figures, Elaine Abraham, George Ramos and David Ramos for their thoughtful advice and support.

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Isoëtes ×*herb-wagneri*, an Interspecific Hybrid of *I. bolanderi* × *I. echinospora* (Isoëtaceae)

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Abstract.—*Isoëtes* ×*herb-wagneri*, the interspecific hybrid between *Isoëtes bolanderi* and *I. echinospora*, is described. The epithet commemorates Warren (Herb) Wagner for the direction and help he provided to the author.

It can be difficult to find one's niche, but Herb Wagner made it easy for me. I met Herb during an American Fern Society foray at the University of Michigan Field Station near Pellston, Michigan in August 1977. At the time, Herb and I had just finished collaborating on a paper about *Dryopteris* ×*leedsii*. Although I had seen him at several gatherings, we had never formally met. I recall our first meeting. For some reason, I thought Herb would recognize me because we had corresponded by phone and mail about *D.* ×*leedsii*. I spotted him standing by the pay phone at the field station the first evening we assembled for the field trip. I approached, smiled, and gave him a cheery "Hi, Dr. Wagner". Herb paused, stared directly at me, and then said, "excuse me, but who the h_ _ _ are you?" Stammering, I mentioned the *D.* ×*leedsii* paper and my name. Herb immediately smiled, shook my hand energetically, and then paid me a wonderful compliment. "I thought you were much older," he said. To my mind, of course, he was implying that, based on our correspondence, he thought I had the wisdom and knowledge of a much older person. During the two-day field trip Herb gave me lots of attention and opened my eyes to reticulate evolution. He reinforced his words with enthusiastic hunts for *Dryopteris* hybrids. Each time I found a hybrid, Herb was right there to excitedly praise and encourage me. I was overwhelmed by his interest and enthusiasm for ferns and for me. By the end of the foray, I knew I would be studying pteridophyte evolution. The following summer, I told Herb about my fascination with *Isoëtes* and how much I enjoyed hunting them. A short while later he told me, "I want you to spend the rest of your life studying quillworts. I'll help you." Never had I received such clear direction. After that Herb created all kinds of opportunities for me to study *Isoëtes*. He made contacts for me. He told others about my interest in the genus. Herb even arranged for a helicopter to take me to the summit of Mount Eke on the Hawaiian Island of Maui to collect a quillwort he and I would later name and describe.

The following *Isoëtes* hybrid is named to honor Warren (Herb) Wagner, for whose direction and help I will always be grateful.

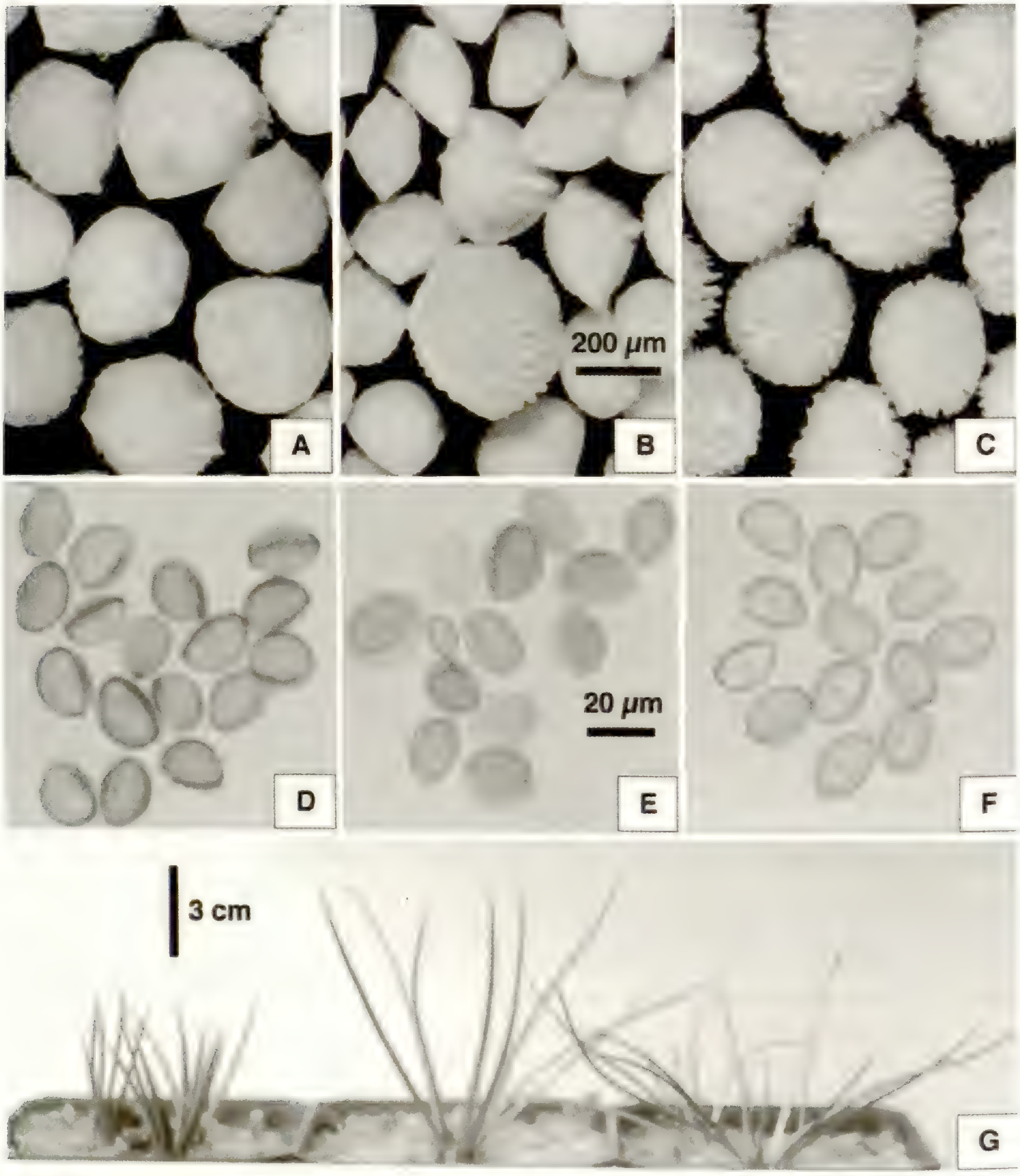

FIG. 1. Megaspores, microspores, and a living plant in cultivation of *Isoëtes* ×*herb-wagneri* and its parents. A–C. Megaspores. A. *I. bolanderi*. B. *I.* ×*herb-wagneri*. C. *I. echinospora*. D–F. Microspores. D. *I. bolanderi*. E. *I.* ×*herb-wagneri*. F. *I. echinospora*. G. *I. bolanderi* (left), *I.* ×*herb-wagneri* (center), *I. echinospora* (right).

Isoëtes ×**herb-wagneri** W. C. Taylor, *hybr. nov.*—Type: United States: Montana: Ravalli Co.: from the bed of the upper of Twin L Lakes, near edge of the autumn level, about 12 ft below the summer level, Lost Horse Canyon, 12 October 1940, *Fred A. Barkley & Reuben A. Diettert 4804* (US 1786885). A

mixed collection of three taxa annotated by W. Carl Taylor as (a) *I. bolanderi*, (b) *I. echinospora*, (c) *I. bolanderi* × *echinospora*, (d) sterile plant. **Fig. 1**

Planta hybrida inter *Isoëtem bolanderi* et *I. echinosporam*, aliis characteribus inter parentes intermedia, sporis male formatis.

Isoëtes ×*herb-wagneri* has been collected only from the type locality at Twin L Lakes. Water is drawn from the adjoining lakes during the summer months, so that by fall, when water levels are low, *Isoëtes* plants are exposed along the margins of the lakes, rooted in a gravelly, clay soil. These exposed plants are a mixture of *I.* ×*herb-wagneri* and *I. bolanderi*. *Isoëtes echinospora* occurs slightly deeper in the lakes, still submerged in the fall, and scattered in stands where the mineral soil is combined with more organic sediments that have accumulated in places.

Isoëtes ×*herb-wagneri* can be recognized by its irregular megaspores that vary in size, shape, and surface ornamentation (Fig. 1B). In contrast, the more uniform spores of *I. bolanderi* and *I. echinospora* are tuberculate or echinate, respectively (Fig. 1A, C). *Isoëtes* ×*herb-wagneri* has characters intermediate between its parents. For example, the uniform microspores of *I. bolanderi* and *I. echinospora* (Fig. 1D, F) are brown or gray, respectively. The irregular microspores of *I.* ×*herb-wagneri* (Fig. 1E) are a combination of brown, light brown, and gray. In addition, the leaves of *I. bolanderi* taper abruptly to a bristle-like apex, whereas those of *I. echinospora* taper gradually to the apex. The leaf apices of *I.* ×*herb-wagneri* are intermediate for this character (Fig. 1G).

Paratype:—United States: Montana: Ravalli Co.: Upper Twin L Lake, 9 October 1989, *David Bilderback s.n.* (MIL).

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Botrychium alaskense, a New Moonwort from the Interior of Alaska

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ABSTRACT.—*Botrychium alaskense* W. H. Wagner & J. R. Grant is described as a new species from the interior of Alaska. It is an allotetraploid of *B. lunaria* (L.) Sw. × *B. lanceolatum* (S.G. Gmel.) Ångstr., the same species parentage that gave rise to the morphologically and isozymically distinct *B. pinnatum* H. St. John.

Outside of North America, only seven species of *Botrychium* are currently recognized: *Botrychium boreale* Milde, *B. lanceolatum* (S.G. Gmel.) Ångstr., *B. lunaria* (L.) Sw., *B. matricariifolium* (Döll) A. Braun, *B. multifidum* (S.G. Gmel.) Rupr., *B. simplex* E. Hitchc., and *B. virginianum* (L.) Sw. Each of these species is also recorded in North America, although the identity of *B. boreale*-like plants in North America is in question, and likely represent either *B. pinnatum* H. St. John or *B. alaskense* W.H. Wagner & J.R. Grant. In addition, North America has more than 25 endemic species, nearly all of which have been recognized in the past three decades, and some of which are still undescribed. A surprisingly large number of these occur in the cordilleran region of western North America.

The majority of the North American endemics have been overlooked apparently because of their inconspicuous appearance, their common occurrence in unexpected, disturbed habitats, or their general rarity. We here describe another new species, this one from the interior of Alaska.

Botrychium alaskense W. H. Wagner & J. R. Grant, *sp. nov.*, **Figs. 1–2.**

A *B. pinnato* H. St. John tropophoro oblongo-deltato, saturate viridibus, pinnis usque 4–6-jugis irregulariter decompositis, inter se approximatis vel distantibus, lobulis plerumque oblongis angulatis, basi anguste (per 40–90°) cuneatis diversa.

→

FIG. 1. Three species of *Botrychium* from northwestern North America. Bottom row left (3 specimens), *Botrychium pinnatum* (*W. H. Wagner 99128 et al.*, MICH). Bottom row right (3 specimens), *B. lanceolatum* (*W. H. Wagner 99120 et al.*, MICH). Remaining (18 specimens), *B. alaskense* (*W. H. Wagner 99005 et al.*, MICH).

TYPE: U.S.A. ALASKA. BIG DELTA QUAD: Vicinity of Fairbanks: Salcha River, 12 km. up the river from its crossing of the Richardson Hwy, at mile 323.1, 64°29′195″N, 146°39′629″W, 23 June 1999, *W. H. Wagner 99005* with *J. R. Grant, F. S. Wagner, A. Gilman, P. Zika, H. W. Grant* and *W. L. Grant* (holotype MICH; isotypes ALA, B, BM, F, G, GH, ISC, MO, NY, P, S, U, UC, US, WTU).

Trophophore stalk 3–10 mm long, up to 2 times the length of the trophophore rachis; lamina bright green, leathery, oblong-deltate, 1-pinnate, up to 6 cm long and wide; pinnae up to 6 pairs, horizontal to slightly ascending, approximate to distant, the basal and supra-basal pair not or slightly more distant than the supra-basal and 3rd pairs, the basal pinnae equal to or slightly longer than the supra-basal pair, broadly lanceolate to broadly trullate, unlobed (small plants) to deeply lobed (large plants), with up to 5, narrowly oblong pairs of lobes, these up to 10 mm long, 3 mm wide, asymmetrically truncate, broadly (2–3 mm) attached and confluent to the rachis, shallowly concave at the basiscopic base; lobe tips irregularly crenate-dentate. Sporophore 2-pinnate, with 3 major branches. Chromosomes evidently tetraploid.

PARATYPES: U.S.A. ALASKA. **ANCHORAGE QUAD**: Black Spruce Campground, Fort Richardson Military Reservation northeast of Anchorage, disturbed floodplain with graminoids and forbs among young cottonwood and alder, 1 August 2001, *D. Farrar 01-07-01-4*. **BIG DELTA QUAD**: Salcha River, 12 km up the river from its crossing of the Richardson Highway at mile 323.1, 64°29′N, 146°45′W, July 1996, *J. R. Grant 96-02625b* (ALA); Salcha River, 12 km up the river from its crossing of the Richardson Highway at mile 323.1, 64°29′N, 146°45′W, 23 June 1999, *J. R. Grant 99-03572* with *W. H. Wagner, F. Wagner, P. Zika, A. Gilman, H. W. Grant, & W. L. Grant* (ALA); Between Harding Lake and the Salcha River on the Richardson Hwy (Alaska Hwy. 2), opposite Harding Rd. [the entrance to the Harding Lake Recreation Area], 64°26′N, 146°54′W, 255 m, 26 June 1999, *W. H. Wagner 99119* with *J. R. Grant, F. S. Wagner & P. Zika* (MICH); Between Harding Lake and the Salcha River on the Richardson Hwy (Alaska Hwy. 2), opposite Harding Rd., 64°26′N, 146°54′W, 255 m, 26 June 1999 *J. R. Grant 99-03584* (ALA); Salcha River, 12 km up the river from its crossing of the Richardson Highway at mile 323.1, 64°29′N, 146°45′W, July 2000, *J. R. Grant 00-3828* (ALA); Between Harding Lake and the Salcha River on the Richardson Hwy., opposite Harding Rd., 64°25′N, 146°53′W, 12 July 2000, *J. R. Grant 00-3833* (ALA); Between Harding Lake and the Salcha River on the Richardson Hwy., opposite Harding Rd. 64°26′N, 146°54′W, 12 July 2000, *J. R. Grant 00-3837* (ALA); Mile 323.1 Richardson Hwy where it crosses the Salcha River, dry disturbed area in pioneer vegetation along roadside, 64°28′N, 146°55′W, 24 June 2001, *J. R. Grant 01-4093* (ALA); Between Harding Lake and the Salcha

→

FIG. 2. *Botrychium alaskense* W. H. Wagner & J. R Grant (drawn from *Grant 00-3833* and *Grant 00-3837*.

Botrychium alaskense

TABLE 1. Comparison of full-sized trophophores (and sporophores) of *Botrychium pinnatum* and *B. alaskense*

Character	*B. pinnatum*	*B. alaskense*
Trophophore outline	Narrowly ovate	Broadly ovate-deltate
Apex	Abruptly narrowed	Gradually narrowed
Pinna overlap when present	Mainly near pinnae bases	Mainly submedial on pinnae, commonly absent
Lowest pinna length	Mostly 2.0 cm or less	Mostly 2.5 cm or less
Lowest pinna width	Mostly 1.0 cm or less	Mostly 1.5 cm or less
Lobe apices	Strongly rounded	Angular
Pinna base angle	120°–140°	40°–90°
Contracted pinna base	Less than 0.5 mm, broadly adnate	At least 1 mm, narrowly adnate
Veinlet interval	Nearly 1 mm	Mostly 0.5 mm or less
Sporophore length	0.5–1.5× trophophore length	0.4–1.0× trophophore length
Low, widely separated sporophore branches	Occasional	Prevalent

River on the Richardson Hwy., opposite Harding Rd., 64°26′N, 146°54′W, 26 June 2001, *J. R. Grant 01-4096* (ALA). **LAKE CLARK QUAD**: North Rim of the Western Amphitheater of the Northern Telaquana Badlands, Lake Clark National Park, in a basin excavated by wind, south facing slope at 5 degrees, vegetation 50% vascular, 50% lichen, 15 August 2001, *P. Caswell s.n.* **LIVENGOOD QUAD**: White Mts., limestone ridge in a grassy slope, 800 m, 15 July 1953, *O. Gjaerevoll 593* (ALA). **MT. McKINLEY QUAD**: Kantishna Mining District, 63°31′N, 150°56′W, 900 m, Wickersham Dome, moist herbaceous, NE-facing alpine slopes, 26 June 1990, *C. J. Parker 2294* (ALA). **NENANA QUAD**: Tamarack Inn, mi. 298, Parks Hwy. [Alaska Hwy. 2], 7 miles S of Nenana, 64°28′N, 149°03′W, 25 June 1999, open field with *B. lanceolatum* (rare), *B. lunaria*, and *B. minganense*, *W. H. Wagner 99113* with *J. R. Grant, F. S. Wagner, & P. Zika* (MICH); *J. R. Grant 99-03578* (ALA). **TALKEETNA QUAD**: Chedotlotina Glacier, Denali National Park, with netleaf willow, mountain avens, polar willow, dwarf scrub meadow, 20 August 2001, *M. Duffy MD-01-258A*. **TANANA QUAD**: Manley Hot Springs area, 21 km E of Manley, 65°05′N, 150°19′W, roadside ditch near Extensive Survey Stand No. 138, 16 June 1973, *J. Foote 3049* (ALA).

Herbarium records from western Alaska and Siberia may also represent *Botrychium alaskense*, but require further study to confirm their identity. The new species may be distinguished from its closest relatives by the following key:

1. Trophophore deltate; sporophore stalk 1/3–1/4 as long as the sporophore; pinna pairs 3 or 4; basal pinna pair 2× as wide as the adjacent pinna pair; basiscopic pinna margin straight to slightly concave and upswept . . . . . *B. lanceolatum*
1. Trophophore ovate to deltate; sporophore stalk 1/2–1/3 as long as the sporophore; pinna pairs 5–7; basal pinna pair equal to or slightly larger than the suprabasal pair; basiscopic pinna margin concave to deeply concave and spreading . . . . . 2.

2(1). Trophophores mostly ovate-deltate; pinnae somewhat irregularly incised, bright green, ovate, distant to approximate, their lobes mostly narrowly oblong, angular; pinna bases forming an angle of ca. 40°–90° . . . . . *B. alaskense*

2. Trophophores mostly ovate; pinnae very regularly incised, dark (olive) green, broadly oblong, mostly approximate, their lobes mostly broadly ovate, rounded; pinna bases truncate to cordate, forming an angle of ca. 120°–140° . . . . . *B. pinnatum*

From plants analyzed using enzyme electrophoresis, it was determined that *B. alaskense* is an allotetraploid of *B. lunaria* × *B. lanceolatum* (D. Farrar, pers. comm.). That would not be a particular surprise, except that *B. pinnatum* has the same parentage. Farrar's preliminary results indicate that *B. alaskense* may have arisen through hybridization between *B. lanceolatum* × *Eurasian B. lunaria*, whereas *B. pinnatum* originated as *B. lanceolatum* × *American B. lunaria*.

Most of the localities where moonworts have been found in interior Alaska include *Botrychium alaskense*; the others observed to date have only *B. lunaria* and/or *B. minganense*. All sites with *B. alaskense* studied by one or both of us are in open, non-forested areas. They include revegetating sandbars along the Salcha River, a mowed field or lawn ca. 30 years old along the Parks Highway, and roadsides along the Richardson Highway.

The immediate vicinity of Fairbanks has produced seven species: *B. alaskense*, *B. lanceolatum*, *B. lunaria*, *B. minganense* Victorin, *B. multifidum*, *B. pinnatum*, and a new species, *B. yaaxudakeit* Stensvold & Farrar. A possible eighth species with affinities to *B. alaskense* may be another undescribed species that is currently being investigated by the junior author. As presently known, the Fairbanks area has the largest number of species of *Botrychium* in Alaska. The most common are *B. lunaria* and *B. minganense*, and the least common is *B. multifidum*. *Botrychium lunaria* was the only species in several localities, while *B. minganense* appeared as the only species in one locality. *B. lunaria* and *B. minganense* grew together in all localities except the foregoing. *B. alaskense*, *B. lunaria*, *B. minganense*, and *B. lanceolatum* were found growing together in three localities, and all seven species were found growing together in one locality.

In three of the sites, the individuals of *B. alaskense* are estimated at well over 200. The plants are obvious because of their unusually large size, characteristic trophophore shape, and shining, bright green color. Growing with *B. alaskense* are young woody plants or herbs. Associated woody plants include *Arctostaphylos uva-ursi*, *Betula neoalaskana*, *Picea glauca*, *Populus balsamifera*, *P. tremuloides*, *Rosa acicularis*, *Rubus idaeus*, *Salix alaxensis*, *S. bebbiana*, *Shepherdia canadensis*, and *Vaccinium uliginosum*. The most prominent associated herbs that can be used as indicators of moonwort sites, even when *B. alaskense* is absent include *Taraxacum officinale*, *Fragaria virginiana*, *Potentilla norvegica*, *Cornus canadensis*, and *Galium boreale*. Particularly, the presence of strawberries nearly always indicates the presence of moonworts.

Botrychium alaskense always grows in somewhat recently disturbed areas. In naturally disturbed sites, it is found on re-vegetating sandbars and along

new oxbow lakes. In man-induced disturbances, populations are found in abundance growing in weedy, infrequently-mowed fields or lawns, or in the ditches and associated edges of the major highways of the interior of Alaska.

The phenology of *B. alaskense* is intermediate between *B. lunaria* and *B. lanceolatum*. *Botrychium lunaria* sporulates first and is completely finished before the sporangia of *B. lanceolatum* have begun to ripen. *Botrychium alaskense* sheds its spores after *B. lunaria* has finished and before those of *B. lanceolatum*.

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A New Name for an Old Fern from North Alabama

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ABSTRACT.—The varieties of *Thelypteris pilosa* have been recognized as the sole New World members of the subgenus *Stegnogramma*. Ferns of this species complex are common throughout central and southern Mexico, Guatemala, and Honduras and exhibit an intriguing temperate disjunction in Alabama. A significant amount of morphological variation exists in the Mexican taxa; it is unclear whether these differences are due to phenotypic plasticity or genetic factors. Two regionally sympatric morphotypes, varying from deltate to lanceolate fronds, occur throughout Mexico and have been described as var. *major* and var. *pilosa*, respectively. A more distinct type, described as var. *alabamensis*, is endemic to north Alabama rockhouse habitats and has been reported from only a single county. Data on ecology, spore morphology, gametophyte biology, and gross frond morphology support the elevation of *T. pilosa* var. *alabamensis* to specific status under the proposed name of *T. burksiorum*.

The year 2002 marks the 160th year since the publication of Martens and Galeotti's *Mémoire sur les fougères du Mexique*. This monumental work represented the first attempt to catalogue the ferns of Mexico and thus introduced several new fern species to science, including *Thelypteris pilosa*, which they discovered in the state of Oaxaca (Martens & Galeotti, 1842, p. 27). This taxon has been recognized as the sole New World member of the obscure, largely Old World *Thelypteris* subg. *Stegnogramma*. Over one hundred years later, Crawford (1951) discovered a single population of *T. pilosa* in Alabama and initiated the first examination of the New World representatives of subg. *Stegnogramma*. Using a limited set of morphological characters, Crawford recognized three varieties, two of which occur throughout Mexico, Guatemala, and Honduras, and the third consisting of the Alabama plants. He applied the name *T. pilosa* var. *pilosa* to a lanceolate morphotype, which happens to correspond with the line drawing rendered by Martens and Galleotti, and *T. pilosa* var. *major* to a deltate morphotype. The more distinct and disjunct Alabama plants, characterized by their much smaller fronds and obtuse pinna apices, he recognized as *T. pilosa* var. *alabamensis*. This segregation of the Mexican varieties has been challenged by Smith (1981, p. 236), while Iwatsuki (1964) recognized Crawford's original varieties. It remains unclear whether these taxa are sufficiently distinct to be recognized as varieties, warrant elevation to specific rank, or are simply extremes within a morphological cline. Because both varieties major and pilosa are common, occur sympatrically in Mexico, and intergrade morphologically throughout their distribution in Mexico, the question of their genetic

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distinction remains unresolved. Here, we discuss the unique morphology and biology of Crawford's third variety, *T. pilosa* var. *alabamensis*, and recognize this taxon as a distinct species.

Thelypteris burksiorum J. E. Watkins and D. R. Farrar, *sp. nov.*—TYPE: U.S.A., Alabama: Winston County, in fissures of Pottsville sandstone, on west fork of Sipsey River 5 miles E of Double Springs, *Watkins 797* (holotype NY; isotypes ISC, UC, UF, UNA). (**Fig. 1**).

Thelypteris pilosa (M. Martens & Galeotti) Crawford var. *alabamensis* Crawford, Amer. Fern J., 41:19. 1951. *Leptogramma pilosa* var. *alabamensis* (Crawford) Wherry, So. Fern Guide 346. 1964. *Stegnogramma pilosa* var. *alabamensis* (Crawford) K. Iwatsuki, Amer. Fern J., 54:142. 1964. TYPE.—U.S.A. Alabama: Winston County: In fissures of Pottsville sandstone, on W fork of Sipsey River 5 miles E of Double Springs, *Crawford 241* (holotype UNA; isotypes UNA, US).

Ex affinitate *T. pilosa* (Martens & Galeotti) Crawford a qua imprimis differt plantae epipetricae. Frondes lanceolatae, 3.5–20 cm. longae; pinnae 0.5–1.5 × 0.5–0.7 cm; laminae, pinnae, et segmenta apice rotundatae.

Rhizomes short-creeping, with lanceolate, reddish brown scales covered with acicular hairs. Fronds fasciculate; stipes 1.5–8 cm long; laminae evergreen, 3.5–20 cm long, 1–3.5 cm wide, often covered with acicular hairs on the veins and laminar tissue, pinnate, lanceolate, tapered to the base, the apex acute and pinnatifid; sori elongate, exindusiate; sporangia with acicular hairs; spores papillate, never spinulose; gametophytes cordate, regularly proliferating, with acicular and copious glandular hairs on the thallus surface.

The species is named in honor of Dr. Robert and Mrs. Mary Burks, who have been voices of conservation in Alabama for decades and who aided in the establishment of the Bankhead National Forest.

Several excellent descriptions of the frond morphology of *T. burksiorum* (Smith, 1993, pp. 217–218) and of *T. pilosa* exist (Mickel & Beitel, 1988, p. 387; Moran & Riba, 1995, p. 193). Sporophytes of *T. burksiorum* differ most notably from *T. pilosa* in their smaller size and abrupt apices. A statistical analysis of 23 quantitative morphometric frond characters found the Alabama plants of *T. burksiorum* to fall outside the range of variation of the Mexican varieties of *T. pilosa* (Watkins, 2000). Following is a more detailed description of the unique spore and gametophyte characteristics of *T. burksiorum*.

Spore Morphology.—*Thelypteris burksiorum* exhibits a spore morphology outside the range of variation of the Mexican material examined (Watkins, 2000). The surface architecture can be described as papillate-verrucose (Fig. 2E, F). The blunt tubercles of the perispore that give this appearance vary in size with an average length of 0.62 mm ($N=15$). These outgrowths were never observed to converge and form connections, as occurs in some specimens from Mexico. The monolete laesura is usually

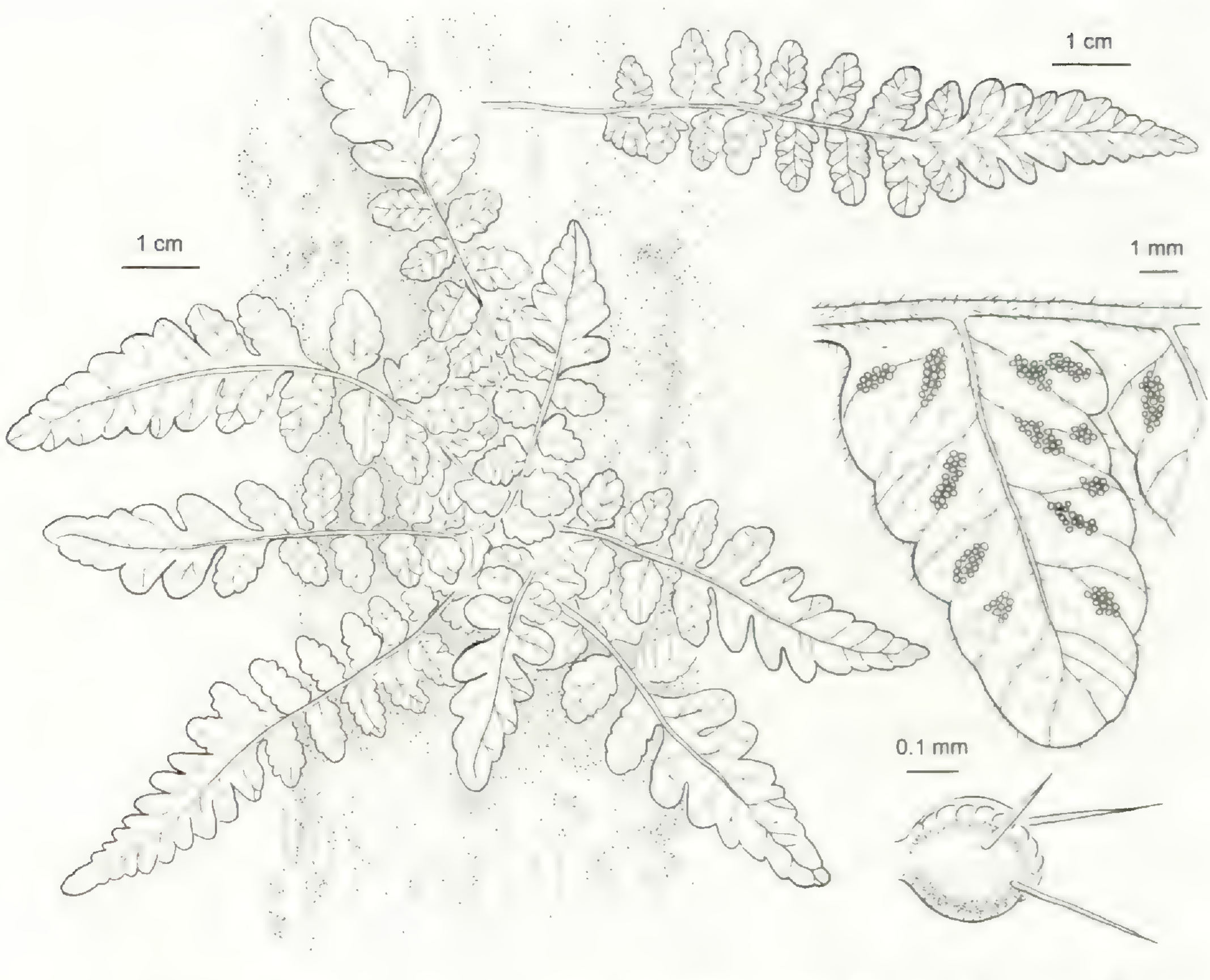
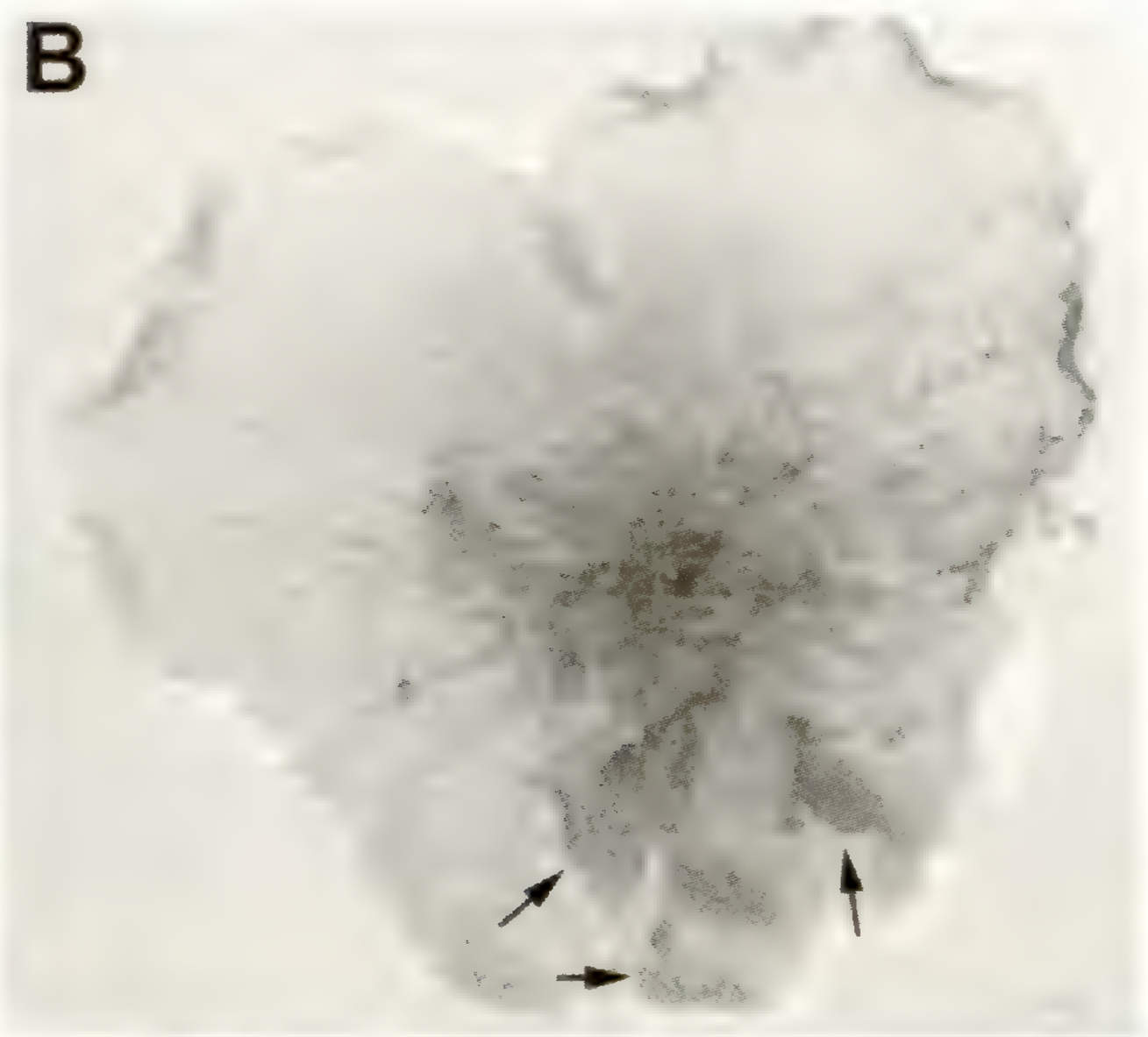


FIG. 1. Sporophytes of Thelypteris burksiorum. Top right. Frond. Middle right. Detail of pinna showing linear, exindusiate sori and acicular hairs on the veins as and laminar tissue. Lower right. Capsule of a sporangium with acicular hairs. Left. Habit of an individual plant growing on

smooth on its surface and free of such ornamentation. The perispore surface is slightly granular on and between tubercles. Mean spore diameter of *T. burksiorum* along the greatest axis is 47 mm ($N=100$), ranging from 44 to 54 mm; along the smallest axis it is 30 mm, ranging from 27 to 36 mm. Mean spore diameter of *T. pilosa* var. *pilosa* along the greatest axis is 46 mm ($N=120$), ranging from 39 to 49 mm; along the smallest axis it is 26 mm, ranging from 25 to 31 mm. Meiotic squashes yielded a base chromosome number of $N=36$, which has been reported to be the base number of *Thelypteris* subg. *Stegnogramma* (Smith, 1993, p. 217).

GAMETOPHYTE MORPHOLOGY.—Gametophytes of *T. burksiorum* are strikingly different from those of *T. pilosa* in hair production, gametangial ontogeny, and gametophyte morphology. Secretory hairs begin to develop at one month along the margin (Figs. 2A, 3A), followed by copious production on the cushion. By two months, the thallus surface and margins are covered by secretory hairs. Development of acicular hairs (Fig. 2A) begins after further maturation (approximately 2.5 months). These are produced sparsely over

A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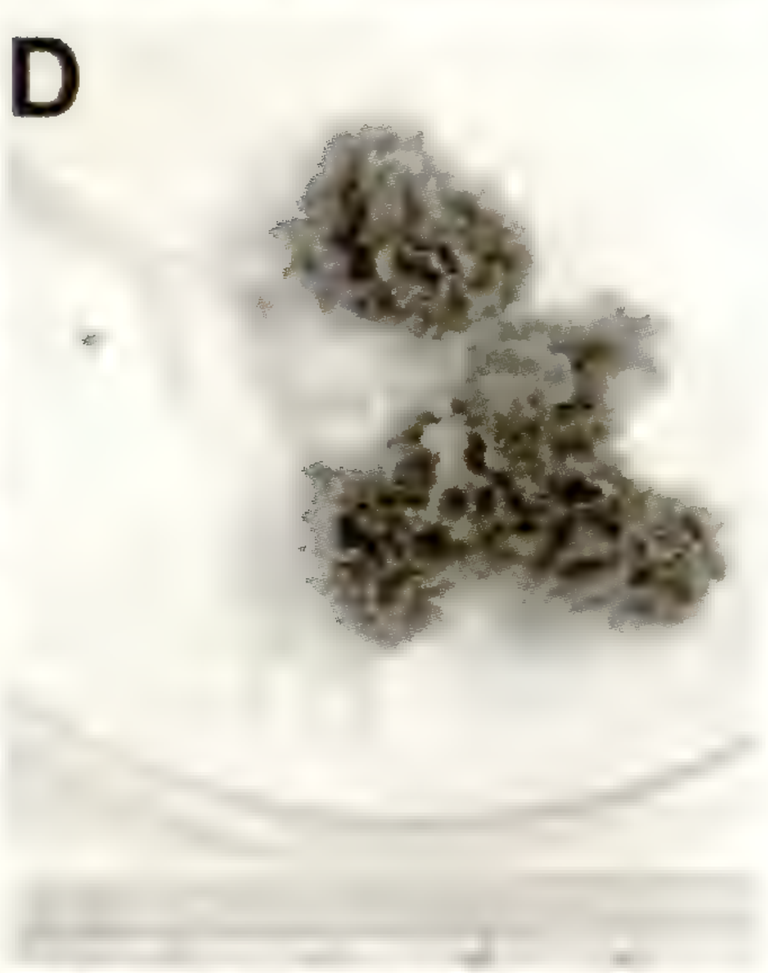
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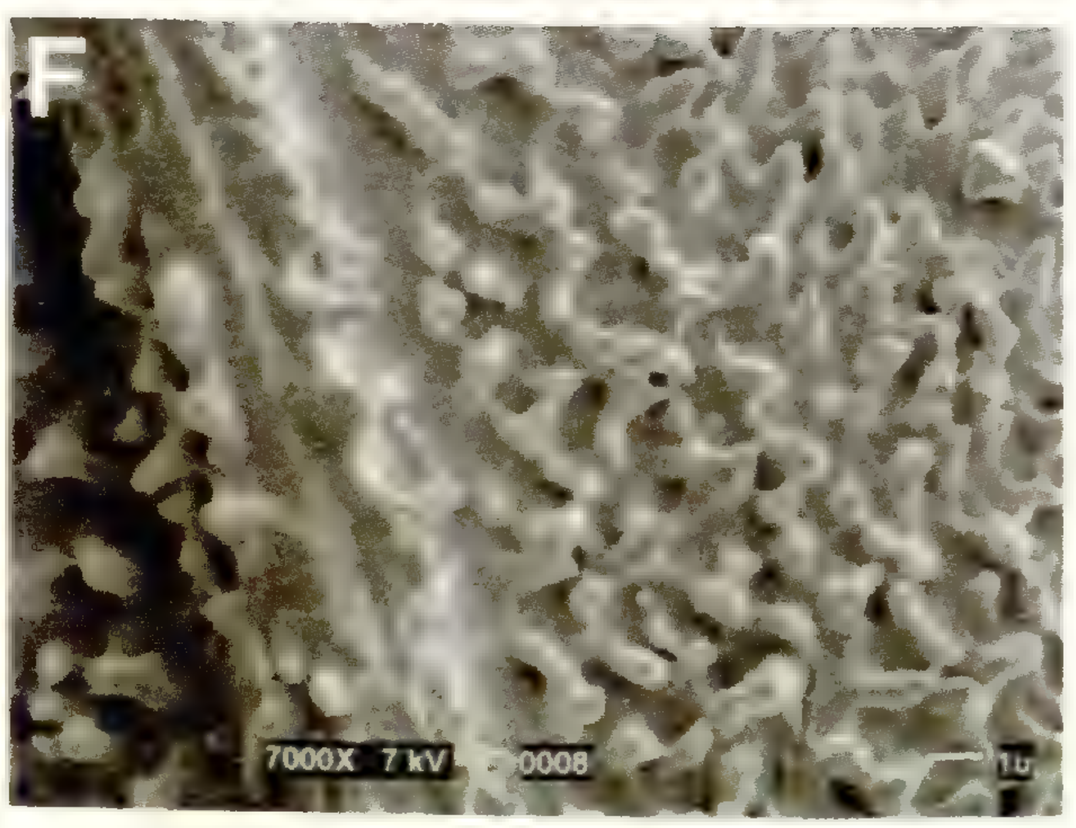
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the archegonial cushion, rarely on the wings, and never along the margins. The gametophyte margins have a ragged-irregular appearance (Figs. 2B–D, 3B), and the surface topography is smooth. In multispore cultures, most gametophytes were without gametangia, but a quantitative determination of gametangium abundance was made difficult by the development of copious gametophytic proliferations described below. For this reason, determining the exact sequence of gametangial development was impossible. Archegonia and swimming sperms (when placed in solution) were visible as early as 2.5 months. All gametophytes that developed gametangia, including those collected in the wild, were hermaphroditic when mature. Attempts to induce cross-fertilization between *T. burksiorum* and the varieties of *T. pilosa* failed to produce sporophytes (Watkins, 2000).

An unusual form of asexual reproduction occurs in gametophytes of *T. burksiorum* that does not occur in gametophytes of Mexican *T. pilosa*. Isolated gametophytes, as well as those in multispore culture, begin to produce proliferations on the dorsal side of the thallus early in development (Fig. 2B, arrows). The proliferations usually begin as filamentous branches that eventually broaden and flatten to form strap-shaped outgrowths (Fig. 3B). Many of these outgrowths eventually take on the appearance of new heart-shaped thalli with pluricellular meristems (Fig. 2C). With experimental severance and transfer to new media, these developed into typical gametophytes that also produced additional proliferations similar to the parent gametophyte. Through proliferation, a single isolated gametophyte, in one year, increased to a size of 3.5 cm and formed a mass of meristematic thalli on its dorsal surface. After 1.5 years of growth, the original thallus still persisted and continued to proliferate (Fig. 2D). Although young, developing proliferations do not produce gametangia while still attached to the parent gametophyte, it seems likely that these proliferations can eventually become independent, producing clonal populations with sufficient gametangia to promote sexual production of sporophytes.

THE UNUSUAL HABITAT OF *THELYPTERIS BURKSIORUM*.—After Crawford's original discovery of *T. burksiorum* in 1952, the taxon went basically unnoticed until bridge-building in the 1960's supposedly destroyed the type and only known locality. It was later rediscovered by Jack Short and John Freeman (1978), and subsequently studied intensively by Gunn in the early 1990's (Gunn, 1990, 1991, 1994, 1996) and by the authors in 1998 and 1999. The species is now known from approximately 17 populations, all of which occur on a 4-mile segment of the Sipsey Fork of the Black Warrior River in Winston

←

FIG. 2. Gametophytes of *Thelypteris burksiorum*. A. Detail of acicular hairs on the thallus margin. B. Mature gametophyte exhibiting irregular margins and early gametophytic proliferations (arrows). C. Proliferations on a year-old gametophyte, some with branches. D. Severed and transplanted proliferations 5 months old showing masses of meristematic thalli. E. F. Typical spore and ornamentation.

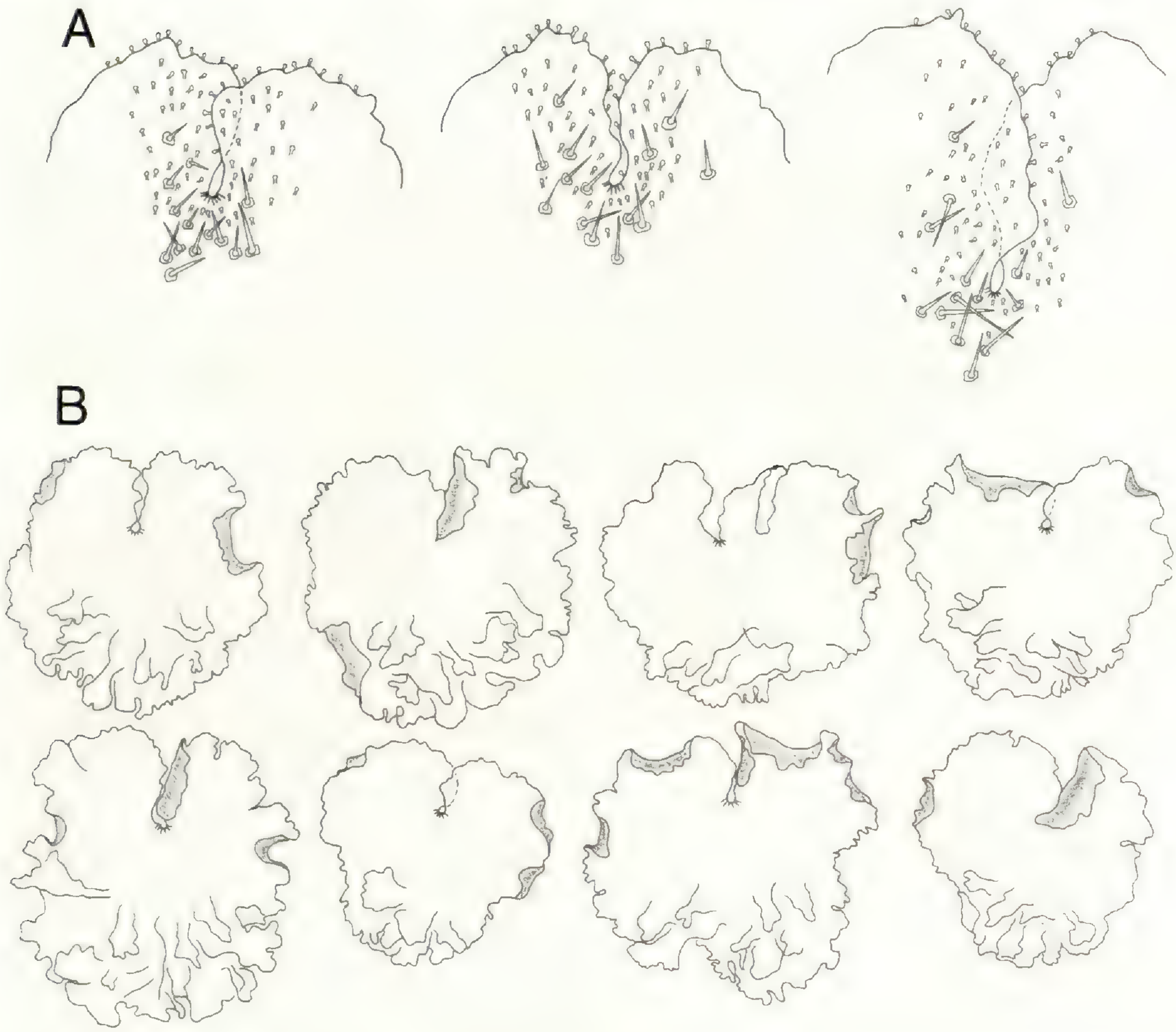

FIG. 3. Gametophytes of *Thelypteris burksiorum*. A. Distribution of acicular and glandular hairs on the thallus. B. Thalli showing asexual proliferations in the basal region.

County, Alabama. This area is characterized by sheer, towering sandstone cliffs composed of Pottsville Sandstone, upon which all known populations of this plant occur (Fig. 4). All plants grow in crevices and rough surfaces on the roofs and floors of sandstone rockhouses formed along these cliffs. Although these rockhouses take on many forms, in the classic sense they are semicircular recesses extending under cliff overhangs (Farrar, 1998; Walck et al., 1996). The key to understanding the microclimates of these intriguing habitats is that they behave much like caves in providing temperature and moisture moderation. In most cases, this creates areas that are always warmer than the surrounding area during the winter and cooler during the summer (Farrar, 1971, 1998). Those rockhouses that have the ability to maintain relatively constant microclimates, combined with a constant supply of water, have been shown to harbor a unique assemblage of tropical bryophytes (Sharp, 1937), tropical pteridophytes (Farrar, 1998), and endemic temperate angiosperms (Walck et al., 1996). It seems probable that *Thelypteris burksio-*

FIG. 4. Habitat of *Thelypteris burksiorum*, which is found only in crevices of Pottsville sandstone along the Sipsey Fork of the Black Warrior River in Winston County, Alabama.

rum, like other tropical members of the rockhouse floras, are relictual from a broader pre-Pleistocene distribution of the species.

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Continued Pteridophyte Invasion of Hawaii

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ABSTRACT.—Two new alien pteridophytes have become established in the Hawaiian Islands since 1996, bringing the total of naturalized alien ferns to 32. Also, established alien species continue to spread onto new islands.

Warren Herb Wagner, Jr. was the first to publish a comprehensive report of the pteridophytes naturalized in the Hawaiian Islands (Wagner, 1950). According to this publication, a total of 21 alien species had become established in the Hawaiian Islands. By 1996 an additional nine naturalized species had been discovered (Wilson, 1996). This paper reports on two newly discovered alien species that have become established, three new records documenting the spread of naturalized species into additional islands, a collection recording the earlier presence of a species than was previously known, and one revised identification of a widespread alien fern. The current status of naturalized ferns and fern allies in Hawaii is shown in Table 1. In the table the species are arranged by the date of their first collection in Hawaii, as determined by the earliest available herbarium collection.

Azolla filiculoides Lam.—In 1943 Fosberg reported *Azolla filiculoides* to have become fully naturalized in taro patches and irrigation ditches on Oahu (Fosberg 1943), after having earlier been deliberately introduced into the Islands as part of a mosquito abatement program. Wagner (1950) reported having collected it on Oahu, as well as on Maui. In 1996, I reported the species to be found from flooded areas on all of the islands except Hawaii, although I speculated that it was also to be found there. A collection by Clyde Imada made in June 1999 from the island of Hawaii now documents its presence there, where he found it growing in a taro farm in Waipio Valley (*Imada 99–16*, BISH).

Blechnum appendiculatum Willd.—The Blechnum species that grows in Hawaii has been known as *B. occidentale* L. since its occurrence was first reported. Recent studies, however, have shown that the rachises of *B. occidentale* are glabrous on the abaxial surface, whereas those of *B. appendiculatum* are pubescent and glandular. *Blechnum appendiculatum* also differs in having more pinnae and darker rhizome scales than does *B. occidentale* (A. R. Smith, pers. comm.; see also Hoshizaki & Moran, 2001, p. 216). The species naturalized in Hawaii is *B. appendiculatum* (syn. *B. glandulosum* Kaulf. ex Link). Both species are natives of tropical America.

Lygodium japonicum (Thunb.) Swartz—A population of *Lygodium japonicum* has been known to be growing north of Hilo, where it was first col-

TABLE 1. Naturalized ferns and fern allies in Hawai'i arranged by year of their first collection. (× = Species recorded on the island).

Species	Year	Kaua'i	O'ahu	Molokai	Maui	Hawai'i
Pteris vittata L.	1887	×	×	–	×	×
Thelypteris dentata (Forssk.) E. St. John	1887	×	×	×	×	×
Pityrogramma austroamericana Domin	1903	×	×	×	×	×
Adiantum raddianum C. Presl	1907	×	×	×	×	×
Macrothelypteris torresiana (Gaud.) Ching	1908	×	×	–	×	×
Pityrogramma calomelanos (L.) Link	1908	×	×	×	×	×
Phlebodium aureum (L.) J. Smith	1909	×	×	–	×	×
Diplazium esculentum (Retz.) Swartz	1910	×	×	–	×	×
Blechnum appendiculatum Willd.	1917	×	×	×	×	×
Ceratopteris thalictroides (L.) Brongn.	1919	×	×	–	–	–
Phymatosorus grossus (Langsd. & Fisch.) Brownlie	1919	×	×	×	×	×
Adiantum hispidulum Swartz	1923	×	×	×	×	×
Nephrolepis multiflora (Roxb.) Jarrett ex C. V. Morton	1923	×	×	×	×	×
Thelypteris parasitica (L.) Fosb.	1926	×	×	×	×	×
Cyrtomium falcatum (L. f.) C. Presl	1928		×	×	×	×
Cheilanthes viridis (Forssk.) Swartz	1928	×	×	–	×	–
Lygodium japonicum (Thunb.) Swartz	1936	–	×	–	–	×
Nephrolepis falcata (Cav.) C. Chr. '*Furcans*'	1936	×	×	×	×	×
Azolla filiculoides Lam.	1937	×	×	×	×	×
Deparia petersenii (Kunze) M. Kato	1938	×	×	×	×	×
Selaginella kraussiana (Kunze) A. Br.	1938	–	×	–	×	×
Angiopteris evecta (G. Forster) Hoffm.	1950	–	×	–	×	×
Lindsaea ensifolia Swartz var. *ensifolia*	1969	×	×	–	×	×
Selaginella stellata Spring	1969	–	–	–	–	×
Cyathea cooperi (W. J. Hook. ex F. Muell.) Domin	1970	×	×	–	×	×
Tectaria incisa Cav.	1972	×	×	–	–	×
Adiantum 'Edwinii'	1981	–	–	–	×	–
Nephrolepis hirsutula (G. Forst.) C. Presl '*Superba*'	1983	×	–	–	×	–
Adiantum tenerum Swartz	1987	–	–	–	×	–
Platycerium bifurcatum (Cav.) C. Chr. subsp. *bifurcatum*	1991	–	×	–	×	×
Marsilea crenata C. Presl	1995	–	×	–	–	–
Salvinia molesta D. S. Mitchell	1999	–	×	–	–	×
Selaginella umbrosa Lemaire ex Hieron.	2000	–	–	–	–	×

lected in 1936 and where it has persisted. Until recently this species is not known to have spread to other sites in Hawaii nor to have become particularly damaging, as it is in the southeastern United States. The status of this species in Hawaii has changed significantly in the last few years. In 1998 George Staples collected it on Oahu, where he found it growing on the

grassy hills above Heeia State Park (Staples 1166, BISH, LAM). Reports have reached me that populations of this species have been seen growing on the island of Hawaii on the hill opposite the Astronomy Department building of the University of Hawaii, Hilo (Palmer, pers. com.), and on Oahu along the Ulu Paina trail, adjacent to Temple Valley, Kaneohe, as well as on another site in the same vicinity (Waters, pers. com.). In 2000, plants were found in Kaneohe, volunteering in gardens among cultivated and native plants (*Gagne s.n.*, BISH #664545, LAM).

Marsilea crenata C. Presl—A well established population of this aquatic was found growing in the demonstration taro patches maintained at the Hawaii Nature Center, Makiki Valley, Honolulu, Oahu. The presence of this population was first recorded on 18 May 1994 in the journal maintained by the Nature Center by Marie Bruegmann. The first collection made at this site was by D. Palmer (*Palmer 2275*, BISH #650412) on 1 June 1995. This taro patch was recently neglected and the water was allowed to drain, but *M. crenata* has persisted in the marshy area, although it does not grow so luxuriantly. Renewed attention to the demonstration taro patch will certainly stimulate the growth of this fern. *Marsilea crenata* is native to the Philippines, Malaysia, Indonesia, Australia, where it grows in shallow fresh water and in drying mud. It is often found in rice paddies. Plants are rooted in the mud, and the leaflets float on the water surface or the erect petioles extend above the surface of the water and hold the leaflets well above the surface. Sporocarps apparently do not develop on the submerged plants, but have been found on a few plants growing along the margins of the taro patch.

Salvinia molesta D. S. Mitchell—This floating aquatic was discovered growing on Oahu in Enchanted Lake, Kailua, and in Lake Wilson, Wahiawa, in April 1999. The earliest collection I have seen of *S. molesta* in the wild was made on 1 April 1999 from the canal and upper end of Kaelepulu Pond, adjacent to Kiukee Place, Enchanted Lakes area, Kailua (*J. Cook s.n.*, BISH). This fern is grown in garden ponds and aquaria and is known to be in cultivation in the Islands. The first collection documenting its occurrence in cultivation in Hawaii was made on 2 July 1991 on Oahu (*Wilson & Staples s.n.*, BISH #606163, LAM). It is also known to be in cultivation on the island of Hawaii. *Salvinia molesta* readily escapes from cultivation and invades bodies of fresh water where it grows aggressively, rapidly becomes a serious pest. Numerous collections were made on Oahu in April 1999, both in the Enchanted Lakes area, Kailua, and in Lake Wilson, Wahiawa, where it has become naturalized. On 15 April 1999 the Honolulu Star-Bulletin reported on planned eradication efforts of this noxious, weedy, aquatic fern. *Salvinia molesta* is native to southern South America (Brazil, Paraguay, and Uruguay) and has become widely naturalized in Africa, Asia, Australia, and New Zealand.

This species has recently also been found in the southern United States, from Texas to Florida, where it is becoming a serious threat to the aquatic systems (Jacono, 1999). It has been declared a Federal Noxious Weed and, as such, it is illegal to own, cultivate, transport, or sell. Every effort needs to be

made to control its growth and spread. *Salvinia molesta* is sexually sterile but reproduces rapidly by fragmentation and is capable of doubling in volume in two to three days to form extensive mats that clog waterways and irrigation canals, block passage, obstruct irrigation pumps, prevent light from reaching aquatic plants, reduce the oxygen content of the water, and seriously degrade the quality of the water (Thomas & Room, 1986).

Selaginella stellata Spring—This species was reported to have been first collected in Hawaii in 1990, but an earlier collection has come to my attention that documents its presence in Akaka Falls State Park, Hawaii, on 30 August 1969 (*Lehto & Lehto 16429*, RSA).

Selaginella umbrosa Lemaire ex Hieron.—This species of *Selaginella* was discovered at Akaka Falls State Park by Kay Lynch on 15 June 2000 (*Lynch s.n.*, LAM). It is found in scattered locations in the area and is not yet widespread. *Selaginella umbrosa*, characterized by having a bright red, unbranched, erect lower stem and a blade-like, flabellate, much branched, bright green upper portion. It is known to be in cultivation in the nearby Hawaii Tropical Botanical Garden. In Hawaii this species is not infrequently found in cultivation, often grown as a ground cover. It is native to from Mexico and Guatemala and south to Colombia. Widely cultivated, it has naturalized in tropical areas including northern Queensland, Australia.

Discussion

The Hawaiian ecosystem continues to be challenged by the invasion of new species of pteridophytes as well as the spread of already present naturalized species. In the six years since the last report on the alien ferns and fern allies in Hawaii (Wilson, 1996), three additional species have been found to be naturalized. *Marsilea crenata* is a known weed in rice paddies in southeastern Asia. In Hawaii it has been found only in one area in a small, cultivated taro plot. There is no information about how it came to be introduced, but it has not been reported to be in cultivation. Its spread in Hawaii is to be anticipated.

Salvinia molesta made its appearance in 1999 and is already well established in different lakes on Oahu. Manual eradication efforts were begun immediately and must be continued if its growth and spread are to be controlled. Biological control projects have not been reported, but these would involve the introduction of insects to the Islands with possible unpredictable consequences. Moran (1992) discussed the biology of this fern as well as the biological control methods that have been used in efforts to prevent its continued spread.

Selaginella umbrosa is not reported to present special problems where it is naturalized. It is widely cultivated, and its continued spread may be anticipated. Cultivated plants in botanical gardens and home gardens provide a rich source for the spread of additional species into the native ecosystem.

Lygodium japonicum is beginning to spread particularly on the windward coast of Oahu, but apparently also on the island of Hawaii. In southern

Alabama and Florida it is weedy and develops a dense growth over the vegetation, thereby shading the underlying plants. Control efforts should begin as soon as possible to protect the Hawaiian flora against a similar growth pattern. *Azolla filiculoides* is now documented to be present on the island of Hawaii. This species is now known to be present on all of the large islands.

The Hawaiian ecosystem continues to be challenged by preridophytes as well as seed plants, and continued efforts to control and eradicate these invaders must be continued.

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I wish to thank George Staples, Daniel D. Palmer, William J. Hoe, Clyde Imada, Brian Aikins, and Brad Waters for continually keeping me apprised of new and interesting pteridophyte discoveries in the Islands. They have notified me of new sightings and have sent me specimens, photographs, and newspaper clippings. David Johnson very kindly confirmed the identification of *Marsilea crenata*. The herbaria of the B. P. Bishop Museum (BISH), Natural History Museum of Los Angeles County (LAM), and Rancho Santa Ana Botanic Garden (RSA) have kindly made their facilities and collections available to me.

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Differentiation of Eastern North American *Athyrium filix-femina* Taxa: Evidence From Allozymes and Spores

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ABSTRACT.—*Athyrium filix-femina* (Lady Fern) comprises a complex of homoploid (n = 40) taxa, distributed over much of the northern hemisphere and extending into South America, whose evolutionary relationships are poorly understood and whose taxonomic treatment is problematic. The *A. filix-femina* complex of North America comprises as many as four taxa with overlapping ranges and provides an especially suitable context for exploring patterns and processes of divergent evolution and its taxonomic consequences in ferns. We addressed differentiation of two eastern North American taxa distinguished on the basis of growth form, frond shape, and spore color, and most recently treated as varieties *A. angustum* and *A. asplenioides* (Northern and Southern Lady Fern respectively). Although, these two taxa have been long perceived as closely related, they have been known to intergrade and recombine to form a hybrid zone in their relatively narrow region of overlap. This perception is supported by the data from the present study. Collections from 17 populations, 9 of *A. angustum* from Quebec to Pennsylvania and 8 of *A. asplenioides* from New Jersey to North Carolina, were examined for spores using LM, SEM, and TEM, and/or allozymes (16 loci coding 10 enzymes). The two taxa exhibited highly distinct perispore surfaces: all *A. angustum* individuals had papillose surfaces, whereas most *A. asplenioides* individuals were rugose with a reticulum of inflated folds. Spores from the northernmost *A. asplenioides* population sampled (Shirley, NJ) showed varying degrees of intermediacy suggestive of introgressive hybridization with *A. angustum*. Levels of allozyme polymorphism in populations (means: P=36.5%, A=1.97, H_E=0.129) were near means for angiosperms and ferns. Genotype frequencies at most loci in all populations were in Hardy-Weinberg equilibrium indicating an outcrossing mating system. Most alleles were shared among all populations. However, at the four most polymorphic loci (*Idh-1*, *Pgi-2*, *Pgm-2*, and *Tpi-2*) allele frequencies were significantly divergent between populations of *A. angustum* and *A. asplenioides*, especially *Idh-1* which approached fixation for alternate alleles. Values for F_{ST} ranged from 0.008 to 0.459 for individual loci (0.255 across loci) with especially high values for *Idh-1*, *Pgi-2*, *Pgm-2*, and *Tpi-2*. Hierarchical F_{ST} analysis indicated that differences between the two taxa (F_{XY}=0.216) accounted for most allele frequency divergence among populations (F_{XY}=0.238). UPGMA analysis of paired Rogers' Similarity (S) values resulted in two principal clusters each comprising populations of one taxon. Populations of *A. angustum* and *A. asplenioides* were joined within their clusters at S=0.938 and S=0.945 respectively, while the two taxon clusters were joined at S=0.848. The spore and isozyme data indicate substantial divergence between *A. angustum* and *A. asplenioides*, suggesting that they merit distinction at the rank of subspecies or species. Additional study of populations in their region of sympatry is required to determine the nature and extent of hybridization.

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Athyrium filix-femina (L.) Roth (Lady Fern) comprises a wide-ranging complex of divergent, homoploid (n = 40) taxa of allopatric or parapatric distribution, variously divided and treated at different ranks (species, subspecies, or variety) by different authors. At least three taxa occur in North America, which Butters (1917) treated as separate species "amply distinct from each other," although he considered *A. filix-femina* from western North America as conspecific with the "true" *A. filix-femina* of Europe. Wherry (1961) followed Butters (1917) in recognizing as distinct species the western *A. filix-femina* (specifying var. *sitchense* Ruprecht), the northeastern *A. angustum* (Willd.) Presl., and the southeastern *A. asplenioides* (Michx.) A. Eaton, although noting that they "intergrade to such an extent as to defy any simple classification." Lellinger (1985) treated these taxa as subspecies of a single globally distributed species, i.e., western *A. filix-femina* ssp. *cyclosorum* (Rupr.) C. Chr., northeastern *A. filix-femina* ssp. *angustum* (Willd.) Clausen and the southeastern *A. filix-femina* ssp. *asplenioides* (Michx.) Hultén. More recently, Kato (1993), stating that "the delimitation and infraspecific classification of *A. filix-femina* need detailed study," treated these taxa at varietal rank, recognizing four North American varieties: in the west, northern *A. filix-femina* var. *cyclosorum* Ruprecht (= var. *sitchense*), a more southerly *A. filix-femina* var. *californicum* Butters (which Butters, 1917 had noted as distinctive but treated as a variety of *A. filix-femina*), and in the east, northern *A. filix-femina* var. *angustum* (Willd.) G. Lawson, as well as a southern *A. filix-femina* var. *asplenioides* (Michx.) Farwell.

These differences in classification reflect neither strongly divergent viewpoints among authors as to the nature of species versus infraspecific taxa, nor differing insights following acquisition of compelling data. Rather, they reflect the best judgment of individual authors in the face of a lack of critical information as to character consistency, degree of intergradation, and genetic relationships among taxa in a group noted for a high degree of variability (Schneller and Schmid, 1982). As a contribution toward elucidating the nature of the taxa composing the *A. filix-femina* complex, the present investigation addressed the distinctness of the two parapatric taxa of eastern North America, *A. filix-femina* var. *angustum* and *A. filix-femina* var. *asplenioides* (referred to henceforth informally as bare epithets *angustum* and *asplenioides* respectively). Characters differentiating these taxa, provided by Butters (1917) and by and large echoed in more recent treatments (Lellinger, 1985; Kato, 1993), include rhizome habit, leaf shape, and notably color and surface features of the spores (Table 1). The goal of the present study was to evaluate more fully the degree to which these putative taxa are distinct by 1) characterizing fine features of the spores using electron microscopy, and 2) surveying allozyme variation across a north-to-south transect through the eastern portion of the range of both taxa.

Materials and Methods

Collections.—All observations were made on plants collected by CLK and CRW from 17 localities, nine *A. angustum* and eight *A. asplenioides* (Table 2).

TABLE 1. Comparison of morphological features of the two putative eastern North American taxa in the *Athyrium filix-femina* complex. Compiled from taxon descriptions in Butters (1917), Lellinger (1985), and Kato (1993).

Character	*A. angustum*	*A. asplenioides*
Rhizome orientation	erect or ascending, more condensed	ascending to creeping, more extended
Scales:	linear lanceolate, 8–10 × 1.5–2 mm, brown to dark brown	lanceolate, 3–9 × 2–3 mm, bronze to light brown or brown
Stipe length relative to lamina	up to half the lamina length	equaling lamina length
Lamina shape	elliptic or rhombic	narrowly deltoid lanceolate, ovate-lanceolate to lanceolate
Frond base	gradually tapered to an acute to obtuse base	slightly reduced and truncate at base
Frond apex	acute to acuminate	acute, acuminate, or more-or-less caudate
Widest portion of lamina	near or just below middle	second pinna pair
Pinna attachment	short-stalked or sessile	usually stalked
Pinna shape	oblong-lanceolate, usually widest at middle and not parallel-sided	oblong-lanceolate to lanceolate, nearly parallel-sided
Pinna apex	acute to acuminate	acute
Sterile v. fertile frond	tending toward dimorphism, the segments of fertile fronds narrower and more acute than those of the sterile fronds	not tending toward dimorphism
Pinnules of fertile fronds	narrowly lanceolate and acute	oblong or linear-oblong and obtuse
Indusium length	tending to be shorter than in *asplenioides*, up to 1.1 mm	tending to be longer than in *angustum*, up to 1.3 mm
Indusium margin[a]	irregularly dentate, and/or ciliate with eglandular hairs	ciliate with glandular or non-glandular hairs as long as indusial width
Sporangial stalks	bearing glandular hairs or less often secondary sporangia	consistently bearing glandular hairs
Spore color	yellowish	brownish-yellow to dark-brown or black
Spore surface	sparsely papillate	wrinkled or reticulated exospore, sometimes nigrescent
Mean spore dimensions	38.6 × 24.7 μ	36.0 × 25.5 μ

[a] Descriptions of the indusium margin in the two taxa are inconsistent in the literature. The table entry is a tentative consensus.

TABLE 2. Collection localities for *Athyrium filix-femina* populations examined in this study. Localities are ordered from north to south.

Population Designation	Collector(s) and collection number	Year Collected	Locality	No. of isozyme samples	No. of spore samples
	A. filix-femina* var. *angustum				
Mt. Sainte Hilaire	*C. Werth s. n.*	1997	Province du Quebec, along hiking trail through forest, Mont Ste.-Hilaire	26	7
Mast Landing	*L. B. Kass s. n.*	1987	Maine, Cumberland County, Mast Landing Sanctuary, Freeport	0	4
North Hudson	*C. Werth and C. Caplen s. n.*	1997	New York, Essex County, *Abies/Thuja* woods and stream bank, 11.3 mi W of North Hudson and I-87, exit 387 to Blue Ridge	26	10
Schenevus	*C. Werth and C. Caplen s. n.*	1997	New York, Otsego County, woods near edge E side I-88, near Schenevus	11	1
Barnet	*C. Werth, D. Conant, and C. Caplen s. n.*	1997	Vermont, Caledonia County, ditches and woods edge along dirt road in vicinity of Conant farm, near Barnet	72	20
Ithaca	*CL Kelloff 560*	1988	New York, Tompkins County, fen along Rt. 96 south of junction with Route 13 approximately 2 miles south of Ithaca	0	20
Binghamton	*CL Kelloff 559*	1988	New York, Broome County, floodplain of Chenango R., 1.5 miles N of Binghamton	0	20
Ralston-1	*CL Kelloff 569*	1988 & 1995	Pennsylvania, Lycoming County, flood plain along Route 14, 7 miles north of Ralston	20	58
Ralston-2[1]	*CL Kelloff 570*	1988	Pennsylvania, Lycoming County, flood plain along Route 14, 3 miles south of Ralston	0*	78

TABLE 2. Continued.

Population Designation	Collector(s) and collection number	Year Collected	Locality	No. of isozyme samples	No. of spore samples
	A. filix-femina* var. *asplenioides				
Shirley	*CL Kelloff 1289*	1997	New Jersey, Elmer County, floodplain along County Road 611, 1.5 miles east of jct. NJ State Route 77, in Shirley	40	27
Breathed Mountain	*CL Kelloff 25*	1987	West Virginia, Tucker County, Breathed Mountains	0	6
George Mason	*CL Kelloff 101*	1988	Virginia, Fairfax County, creekside on George Mason University campus in Fairfax	0*	30
Hopewell	*CL Kelloff 565*	1988 & 1995	Virginia, Fauquier County, floodplain along Route 629, 3.3 miles N of Route 601 near Hopewell	30	42
Mountjoy Store	*CL Kelloff 564*	1988 & 1995	Virginia, Stafford County, bog at Mountjoy Store, junction of routes 611 and 633	30	63
Pond Drain	*C. Werth, E. Potter, and K. Sciaretta s. n.*	1997 & 1998	Virginia, Giles County, north-facing wooded slope along rd. to White Pine Lodge and above Pond Drain, NW of rt. 613 and Mountain Lake	50	0
Pipevine Hollow	*CL Kelloff s. n.*	1987	Tennessee, Davidson County, Pipevine Hollow	0	?5
Sandy Run Swamp	*CL Kelloff 1285*	1997	North Carolina, Onslow County, creek bank and roadside ditch E side of Sandy Run Swamp along Haws Run Road, just W of Padgett, ca. 60 air km NE of Wilmington	32	18

[1] Initial isozyme data were reported for 78 individuals from Ralston-2 (Kelloff 1990), but this population was not resampled during 1995 and isozyme data are not included in the present report (see text for further explanation).

Of these, seven were examined only for spores, while for ten (five of each taxon), population samples for isozyme electrophoresis were obtained as well. At most localities, plants were collected in a transect and individuals numbered successively. Voucher specimens for localities collected by CLK are deposited at the George Mason University Herbarium (GMUF), those collected by CRW at the E. L. Reed Herbarium of Texas Tech University (TTC).

SPORE MORPHOLOGY.—Spores were collected from fresh samples gathered from 16 of the localities listed in Table 2. The spores were examined for surface characteristics of the perispore using light microscopy (LM; 409 individuals), and scanning electron microscopy (SEM; one to two individuals from ten populations, 13 from the Shirley, NJ population). For exine ultrastructure, transmission electron microscopy (TEM) was used to examine four individuals, 2 of each taxon. For LM, spores were mounted by gently tapping a pinnule bearing mature sporangia over a glass slide and then adding a few drops of Permount™ mounting medium and a glass coverslip. Spores were examined under a standard brightfield optic system.

For SEM, air dried spores were affixed to glass coverslips with ethanol, mounted on SEM stubs, and sputter coated with gold or carbon/gold palladium using the Hummer VII sputtering system. The spores were then examined using either a Hitachi S-530 or a LEO 440 scanning electron microscope. From each SEM sample at least 100 spores were examined.

For TEM, each spore sample was embedded in 1% agar as a pellet and fixed in sodium veronal-acetate buffered potassium permanganate (Dawes, 1971). The sample pellet, cut up into 1.0 mm cubes, was dehydrated in a graded series of ethanol. After dehydration, the spores were infiltrated with a series of propylene oxide and Spurrs plastic embedding medium in a labeled BEEM capsule and polymerized in an oven at 70°C for 16 hours. The blocks were then thin-sectioned with a Dupont diamond knife, the sections picked up on #200 mesh grids and examined with a JEOL 100C transmisson electron microscope. Post-staining of the spore sections was not necessary when fixed with the buffered potassium permanganate (Dawes, 1971).

ISOZYME ELECTROPHORESIS.—Fronds to be analyzed electrophoretically were collected in the field and kept refrigerated in plastic bags until homogenized within a few days. For each sample, approximately 1 g leaf tissue was ground using eight drops of "microbuffer" (Werth, 1985) enhanced with 5% PVP-40T and 1% 2-mercaptoethanol. The tissue was ground in a porcelain spot plate using a small test tube as a pestle and sand to facilitate grinding. The extract was either absorbed onto filter paper wicks and used immediately or stored at −78°C for up to four months before being thawed and absorbed onto wicks. Starch gel concentration varied from 11% to 13.5% depending on the properties of individual starch lots.

The following eleven enzymes were analyzed using the buffer systems indicated: on lithium hydroxide (Selander *et al.*, 1971)—glutamate oxaloacetate transaminase (GOT), hexokinase (HK), and leucine aminopeptidase (LAP); on system number 6 (Soltis *et al.*, 1983)—phosphoglucose isomerase

(PGI), phosphoglucomutase (PGM), and triose-phosphate isomerase (TPI); on morpholine-citrate pH 8.2 (Werth, 1991)—aldolase (ALD), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGDH), and shikimate dehydrogenase (SKDH). Gels were run until the bromophenol blue marker migrated 10 cm for lithium-hydroxide and number 6, or 12 cm for morpholine-citrate. Staining followed Werth (1985) as modified for the zymecicle technique (Werth, 1990). Isozyme banding patterns were readily interpreted as allelic variation for single gene loci from whole leaf extracts (cf. Gastony and Darrow, 1983) using standard principles (Wendel and Weeden, 1989; Murphy *et al.*, 1990). Data were analyzed using the BIOSYS-1 computer program (Swofford and Selander, 1981), entered into the program as individual multilocus genotypes. Occasionally, sets of two or three successive samples from the field possessed identical genotypes. As *Athyrium filix-femina* is known to occasionally form clones up to nearly 20 m in extent (Sciaretta *et al.*, ms), such sets were assumed to comprise single genets and each set was represented by only a single entry in the data set.

Initial isozyme data were obtained from four populations, two of each taxon (data reported in Kelloff, 1990). During 1995, three of these populations (Ralston-1, PA, Hopewell, VA, and Mountjoy Store, VA) were revisited and re-sampled, and an expanded isozyme data set obtained; this was augmented by sampling of additional populations during 1997 and 1998. The initial data were largely consistent with the more recent results; however, only the latter are reported here to avoid including inadvertently re-sampled individuals.

Results

Spores, Light Microscopy.—Observation of spores through the light microscope (LM) revealed features largely consistent with previous reports of differences between the two taxa (Butters, 1917; Lugardon, 1971; Schneller, 1989). Spores of both *A. angustum* (Fig. 2a, b) and *A. asplenioides* (Fig. 3a, b) were monolete with an unbranched longitudinal proximal laesura, ovoid to ellipsoidal in polar view, and possessed a perispore (terminology of Erdtman, 1969). Although most of the surface details were obscure when viewed under LM, the difference between the two taxa was readily seen in their outline. The spores of *A. angustum* had a somewhat smooth surface (Fig. 2a, b), while those of *A. asplenioides* had an uneven surface (Fig. 3a, b).

Spore color has been described as differing between *A. angustum* with yellowish spores, and *A. asplenioides* with yellowish-brown to blackish spores (Table 1). This characterization evidently is based on appearance of mass spores under reflected light as viewed unaided or with a dissecting microscope. When viewed under LM using transmitted light, spores of both taxa appeared yellowish, with the exception of occasional *A. asplenioides* spores that displayed an especially dark or blackish reticulation overlaying the yellow surface of the spore.

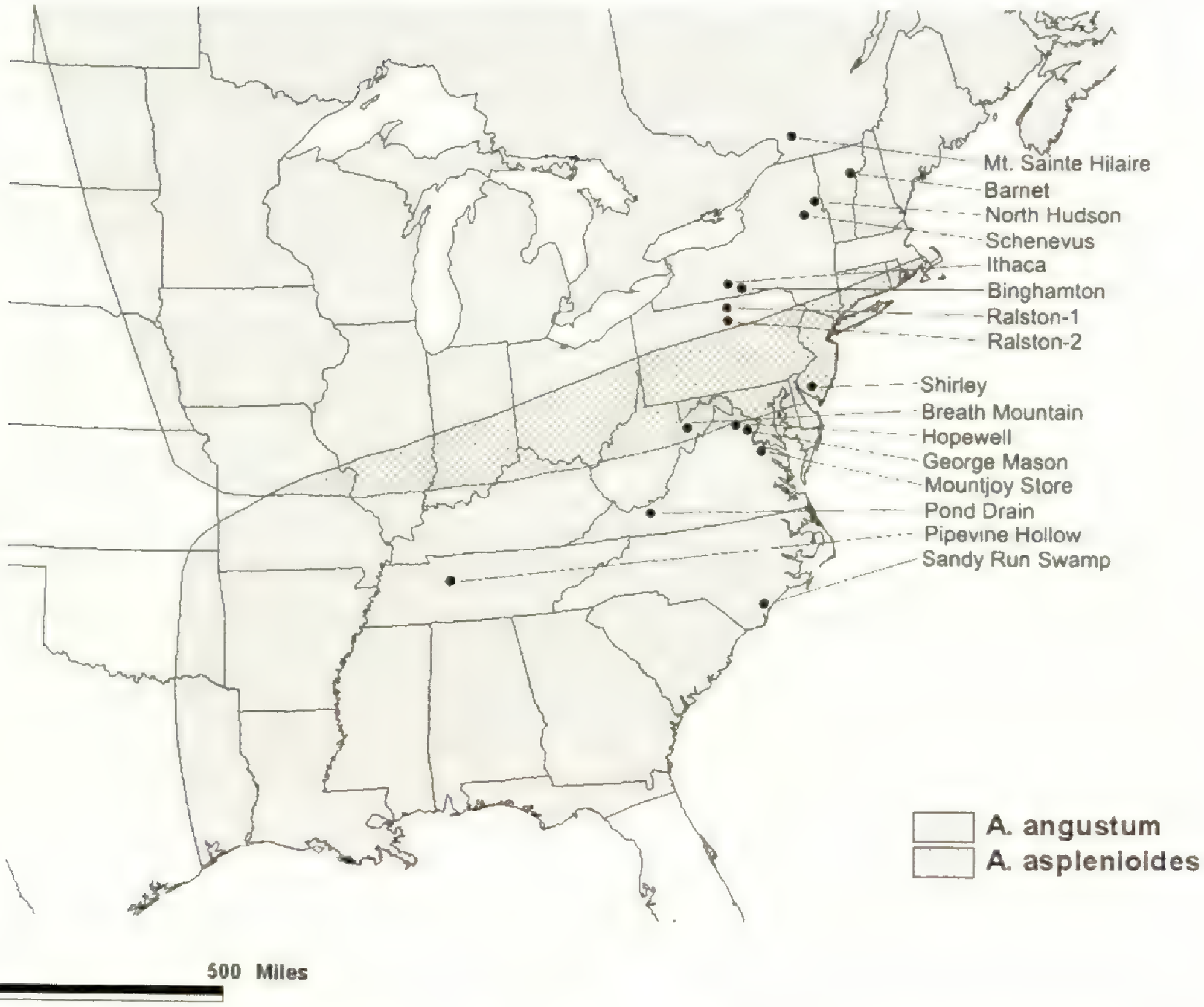

FIG. 1. Map showing overlapping ranges of two *Athyrium* taxa, *angustum* and *asplenioides*, in eastern North America and location of populations studied.

SCANNING ELECTRON MICROSCOPY.—Observations of spores under SEM were consistent with those of Tryon and Lugardon (1991) and Schneller (1989) in revealing that the perispore of *A. angustum* (Fig. 2c–f) differs markedly from that of *A. asplenioides* (Fig. 3c–f). The perispore of *A. angustum* was papillose, its surface completely and evenly covered with minute verruciform (i.e., wart-like) projections. On some spores of *A. angustum*, thin, irregular laminae of perispore material appeared as occasional "flakes." In contrast, the perispore of *A. asplenioides* was rugose, characterized by a network of muri (i.e. ridges/walls) that meandered and joined to surround lacunae. The surface of the lacunae completely lacked the projections as in *A. angustum*.

TRANSMISSION ELECTRON MICROSCOPY.—Under TEM the similarities and differences between the structural and sculptural features of the spore wall of the two taxa were clearly distinct. Both *A. angustum* and *A. asplenioides* (Figs. 2f and 3f) possessed a thick external exospore (Ee). Although not evident in these sections, the fern exospore is composed of feuillettes, i.e., fused plates (Lugardon, 1976, 1979; Tryon, 1986). TEM revealed that the sculptural elements composing the surface details seen under SEM were not derived from

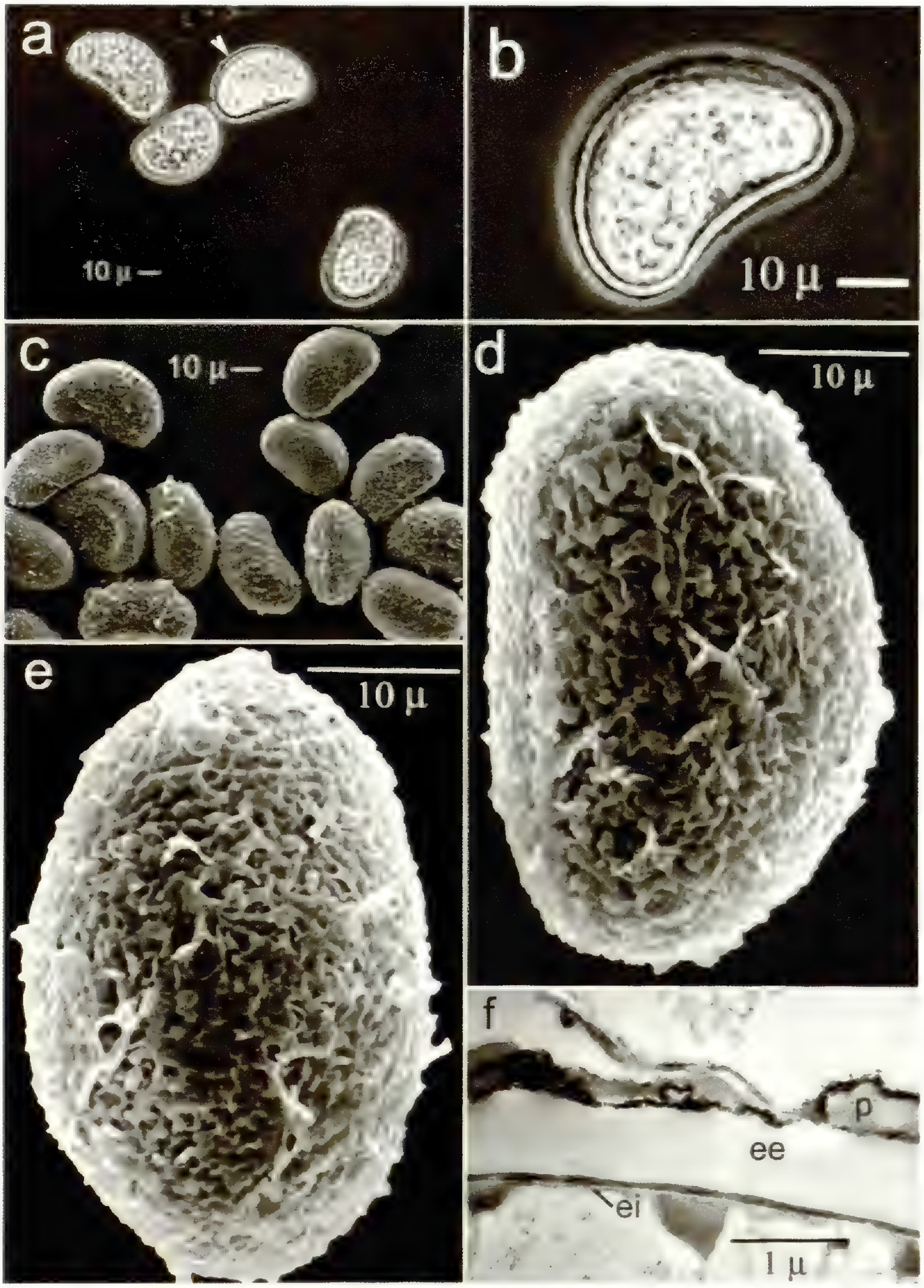

FIG. 2. Spores of *Athyrium angustum*. A, B. Spores viewed under LM. Note smooth appearance of perispore. C, D, E. Spores viewed under SEM. Note papillate perispore surface. F. Spore wall viewed under TEM. Note low perispore (p), external exospore (ee), and internal exospore (ei).

the exospore, as for example in the spores of *Trichomanes* or *Protomarattia* (Tryon, 1986), but instead from the perispore, the outer stratum circumjacent to the external exospore. The perispore of both taxa fit tightly to the external exospore. This stratum in the spores of *A. angustum* (Fig. 2f) was a shallow,

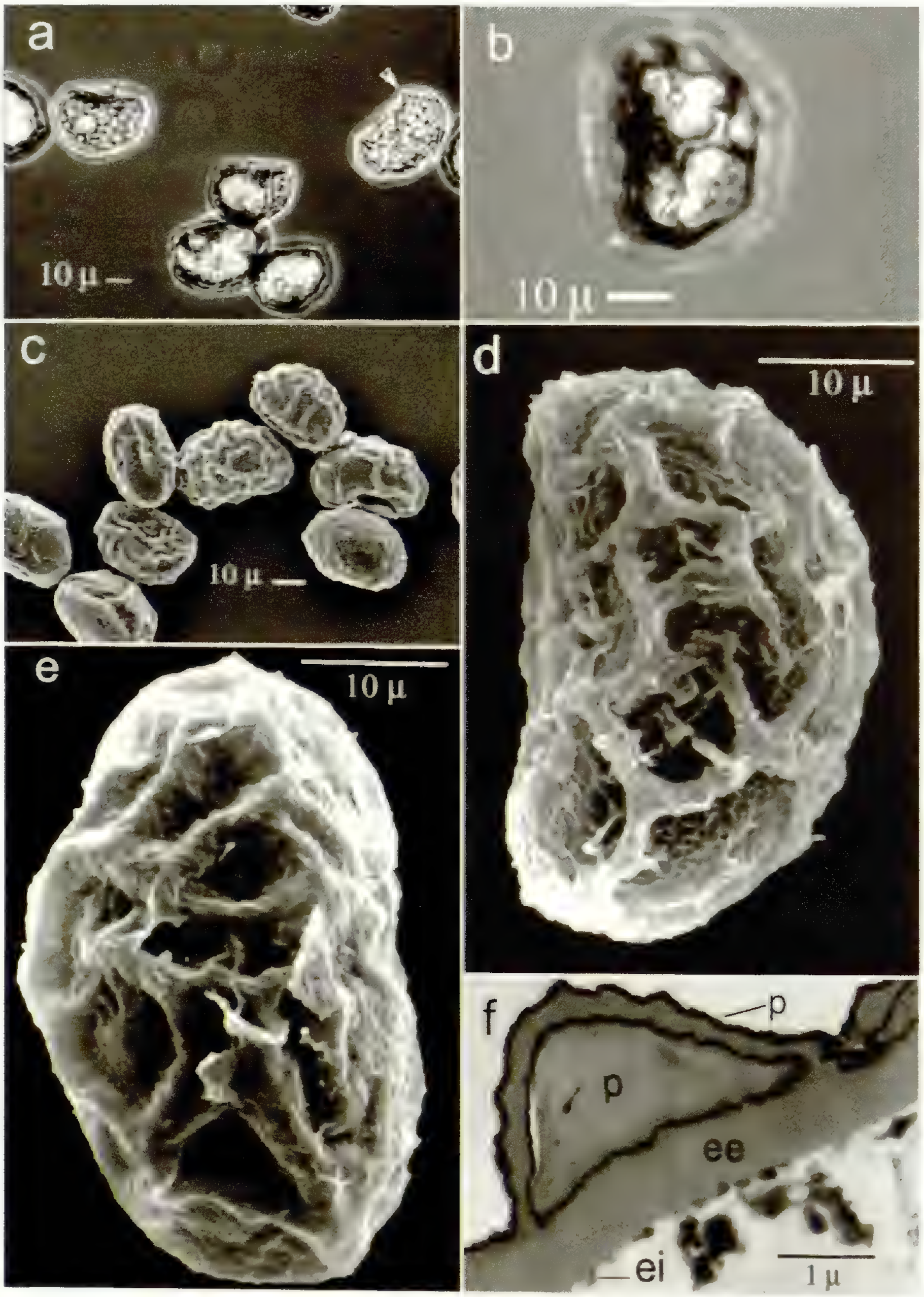

FIG. 3. Spores of *Athyrium asplenioides*. A, B. Spores viewed under LM. Note rugose, reticulate appearance of perispore. C, D, E. Spores viewed under SEM. Note reticulating inflated folds of perispore surface. F. Spore wall viewed under TEM. Note inflated fold in perispore (p), external exospore (ee), and faint internal exospore (ei).

fairly uniform layer. In *A. asplenioides* spores, what appeared to be muri under the SEM (Figs. 3c–e) were more clearly seen under TEM to be inflated folds in the perispore because the columellae, characteristic of true muri, were lacking (Fig. 3f). Beneath the external exospore on *A. angustum* was

a thin endospore or internal exospore (Ei), a layer that is deposited shortly before germination of the spore (Tryon, 1986). A remnant of this layer was also visible in *A. asplenioides*.

TAXON FIDELITY OF SPORE MORPHOTYPES AND EVIDENCE FOR HYBRIDIZATION.—All populations of *A. angustum* sampled for this study exhibited the smooth, micropapillate spore morphotype, and all populations of *A. asplenioides* except one exhibited the rough, rugose spore morphotype. Reliance on light microscopy, which may fail to reveal the subtle detail of the perispore, and on color, which varies in *A. asplenioides* despite the overall similarity of perispore in this taxon, may have led to some confusion in the previous literature. For example, Liew (1971) concluded that most individuals of both *A. angustum* and *A. asplenioides* produced both kinds of spores, entirely inconsistent with our observations.

The exceptional *A. asplenioides* population was the northernmost population sampled, Shirley, NJ. At this site the spores had perispore sculpturing characteristics of both *A. angustum* and *A. asplenioides*. While none of the plants from this site possessed *A. angustum*-type spores, spore arrays of the 13 plants examined under SEM possessed unique and variable morphologies that suggested an influence from *A. angustum* (Fig. 4). The greatest degree of *A. angustum* influence was exhibited by six individuals in which prevailing perispore morphotype was very irregular in appearance, combining an array of sculptural elements that were difficult to characterize (Fig. 4a, b). These included irregularly shaped papillae, briefly-extending isolated muri, and a preponderance of irregular small lamellae reminiscent of the "flakes" seen in *A. angustum*. This prevailing morphotype is readily interpreted as intermediate between the highly distinct morphotypes of *A. angustum* and *A. asplenioides*, seeming to average their disparate surface features and indicating that these individuals may be hybrids between the two taxa. Many of the highly variable spores of these putative F1 hybrids were not entirely intermediate, but rather showed tendencies toward either the smooth *A. angustum* type or the rough *A. asplenioides* type (Fig. 4a, c–g). Spores that fully resembled parental types were discovered in the spore arrays of these six individuals, although these extremes were decidedly rare. The spores of these putative hybrids were not abortive, as is typically the case in interspecific fern hybrids, but rather appeared normal, i.e., well-filled, and were found to be viable as evidenced by successful germination (unpublished data). Moreover, the seven other individuals in the Shirley, NJ, population examined under SEM possessed spore arrays exhibiting various degrees of intermediacy but strongly skewed toward the *A. asplenioides* morphology. These are hypothesized to include backcrosses between first-generation hybrids and *A. asplenioides* (two individuals) and later generation backcrosses (five individuals).

ALLOZYMES, GENERAL.—The eleven enzymes assayed were coded by 17 interpretable loci of which only one (*Pgi-1*) was invariant across all ten populations. The remaining sixteen loci (*Ald, Got, Hk, Idh-1, Lap, Mdh-1, Mdh-2,*

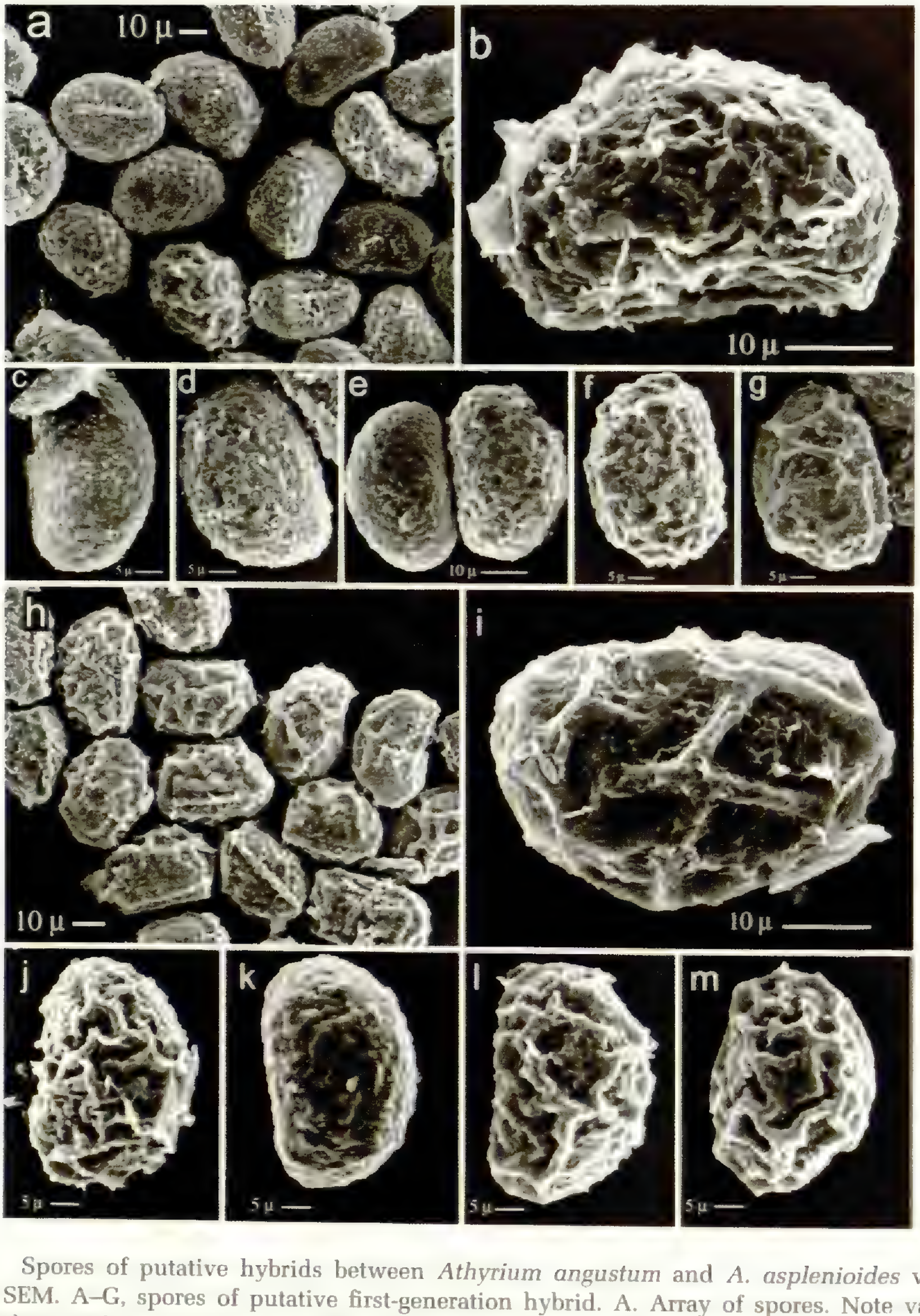

FIG. 4. Spores of putative hybrids between *Athyrium angustum* and *A. asplenioides* viewed under SEM. A–G, spores of putative first-generation hybrid. A. Array of spores. Note variable morphology and preponderance of "flaky" intermediates. B. Close-up of "flaky" intermediate spore morphotype. C–G. Examples of spore morphotypes occurring in putative F-1 hybrid, varying from *A. angustum*-like (C) to *A. asplenioides*-like (G). H–M, spores of putative backcross between F-1 hybrid and *A. asplenioides*. H. Array of spore morphotypes exhibited by the putative backcross. Note prevalent of *A. asplenioides*-like spores. I. Close-up of *A. asplenioides*-like spore from backcross. Note irresolute nature of inflated folds and tendency toward "flakiness". J–M. Examples of spore morphotypes occurring in putative backcross. Note variation from "flaky" intermediate (J) to *A. asplenioides*-like (M).

Mdh-3, *Mdh-4*, *Pgi-2*, *Pgm-2*, *6-Pgd-1*, *6Pgd-2*, *Skdh*, *Tpi-1*, and *Tpi-2*) were variable in at least one population, and the frequencies of the alleles were computed (Table 3). There was a strong tendency for all populations to share the more common alleles and some of the less frequent ones as well. Most of the loci were weakly polymorphic, a single allele predominating in all populations of both *A. angustum* and *A. asplenioides*. In contrast, at the four most polymorphic loci (*Idh-1*, *Pgi-2*, *Pgm-2*, and *Tpi-2*), population allele frequencies were similar within *A. angustum* and *A. asplenioides* respectively, but strikingly different between the two taxa (Table 3).

The most contrasting locus was *Idh-1* for which alleles $Idh\text{-}1^B$ and $Idh\text{-}1^C$ prevailed in *A. angustum* at frequencies ranging from 0.545 to 0.676 and 0.296 to 0.455 respectively, while allele $Idh\text{-}1^A$ was the most frequent in *A. asplenioides* with frequencies ranging from 0.828 to 0.950. At *Pgi-2* both taxa shared $Pgi\text{-}2^E$ as their most frequent allele; however, $Pgi\text{-}2^G$ was represented in all *A. angustum* populations at much higher frequencies, ranging from 0.154 to 0.450, than in populations of *A. asplenioides* for which the frequency of this allele ranged from 0.000 to 0.106. At *Pgm-2*, both taxa shared $Pgm\text{-}2^B$ and $Pgm\text{-}2^C$ as principal alleles, the former prevailing in *A. angustum* populations at frequencies ranging from 0.856 to 1.000, the latter prevailing in *A. asplenioides* populations at frequencies ranging from 0.580 to 0.857. At *Tpi-2* both taxa shared three prevalent alleles, with $Tpi\text{-}2^A$ being of substantial frequency in all populations. Allele $Tpi\text{-}2^B$ occurred at high frequencies, ranging from 0.355 to 0. 550, in *A. angustum* populations whereas $Tpi\text{-}2^C$ was infrequent in this taxon, occurring at frequencies from 0.000 to 0.100. Conversely, $Tpi\text{-}2^C$ often was the most frequent allele in *A. asplenioides* populations, occurring at frequencies from 0.316 to 0.606, while $Tpi\text{-}2^B$ was rarer, occurring at frequencies from 0.000 to 0.255. The contrasting allele frequency trends for these four loci were highly consistent among populations within each taxon. Exceptions to these frequencies trends were in the northernmost sampled *A. asplenioides* population, Shirley, from southern New Jersey for the characteristically *A. angustum* alleles $Idh\text{-}1^B$ and $Pgi\text{-}2^G$ and likewise for $Tpi\text{-}2^B$ in the highest elevation (1300 m) population of *A. asplenioides* sampled, Pond Drain, from the mountains of southwestern Virginia. Moreover, five of the six Shirley, NJ, individuals hypothesized to be first-generation hybrids on the basis of spore morphology were heterozygous for *A. angustum* and *A. asplenioides* marker alleles for at least three of the four most divergent loci, i.e., $Idh\text{-}1^{AB}$, $Pgi\text{-}2^{EG}$, $Pgm\text{-}2^{BC}$ (scored for only two of these individuals) and $Tpi\text{-}2^{BC}$. No other individuals in the entire data set possessed this genotype combination.

GENETIC VARIATION.—Genetic variation was quantified for each population and the species as a whole by computing three standardly used indices. Values for percent loci polymorphic (P) ranged from 23.5% to 47.1%, with a mean of 36.48%; for mean number of alleles per locus (A), the range was 1.5 to 2.5, mean 1.97; and for mean expected heterozygosity (H_E), the range was 0.112 to 0.147, mean 0.129 (Table 4). The mean values for these indices in

TABLE 3. Allele frequencies for 17 isozyme loci in ten populations of *Athyrium filix-femina* in eastern North America.

		angustum					*asplenioides*				
Locus	Allele	Mt. Ste. Hilaire QE	Barnet VT	North Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA
Ald	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.969
	B	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.021
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	(N)	19	29	26	11	1	8	1	1	32	48
Got	A	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	1.000	0.951	1.000	1.000	1.000	0.955	1.000	0.917	1.000	0.980
	C	0.000	0.016	0.000	0.000	0.000	0.045	0.000	0.083	0.000	0.010
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	(N)	10	61	5	6	20	33	30	30	12	50
*Hk**	A	0.000	0.000	0.000	0.000	0.150	0.000	0.017	0.000	0.000	0.016
	B	0.960	0.965	1.000	0.955	0.800	0.868	0.967	0.967	0.893	0.952
	C	0.040	0.023	0.000	0.045	0.050	0.105	0.017	0.017	0.089	0.032
	E	0.000	0.012	0.000	0.000	0.000	0.025	0.000	0.017	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.018	0.000
	(N)	25	43	19	11	10	19	30	30	28	31
*Idh-1**	A	0.019	0.007	0.000	0.000	0.000	0.828	0.950	0.833	0.859	0.920
	B	0.596	0.676	0.596	0.545	0.650	0.172	0.017	0.133	0.094	0.060
	C	0.385	0.296	0.385	0.455	0.350	0.000	0.000	0.000	0.000	0.020
	D	0.000	0.021	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	F	0.000	0.000	0.000	0.000	0.000	0.000	0.033	0.033	0.047	0.000
	(N)	26	71	26	11	20	32	30	30	32	50
Lap	A	0.000	0.000	0.000	-	0.000	0.000	0.018	0.000	0.000	0.000
	B	0.100	0.038	0.063	-	0.175	0.000	0.036	0.000	0.031	0.016
	C	0.850	0.885	0.750	-	0.550	1.000	0.946	1.000	0.938	0.935
	D	0.050	0.058	0.188	-	0.275	0.000	0.000	0.000	0.016	0.032
	E	0.000	0.019	0.000	-	0.000	0.000	0.000	0.000	0.016	0.000
	F	0.000	0.000	0.000	-	0.000	0.000	0.000	0.000	0.000	0.016
	(N)	10	26	16	0	20	33	28	23	32	31

TABLE 3. Continued.

		angustum					*asplenioides*				
Locus	Allele	Mt. Ste. Hilaire QE	Barnet VT	North Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA
Mdh-1	A	0.981	0.950	0.942	1.000	1.000	0.939	0.967	1.000	0.969	1.000
	B	0.019	0.020	0.058	0.000	0.000	0.061	0.033	0.000	0.000	0.000
	C	0.000	0.030	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000
	(N)	26	50	26	11	20	33	15	20	32	50
Mdh-2	A	1.000	0.990	1.000	1.000	1.000	1.000	0.933	0.950	1.000	1.000
	B	0.000	0.010	0.000	0.000	0.000	0.000	0.067	0.050	0.000	0.000
	(N)	26	50	26	11	20	33	15	20	32	50
Mdh-3	A	1.000	1.000	1.000	1.000	1.000	0.985	0.967	1.000	0.953	1.000
	B	0.000	0.000	0.000	0.000	0.000	0.015	0.033	0.000	0.047	0.000
	(N)	26	50	26	11	20	33	15	20	32	50
Mdh-4	A	0.960	0.920	0.962	1.000	1.000	1.000	0.967	0.950	1.000	1.000
	B	0.040	0.070	0.038	0.000	0.000	0.000	0.033	0.050	0.000	0.000
	C	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	25	50	26	11	13	33	15	20	32	50
Pgi-1	A	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	(N)	22	67	21	11	10	13	25	24	27	38
Pgi-2	A	0.000	0.000	0.000	0.000	0.000	0.015	0.033	0.000	0.000	0.000
	B	0.000	0.014	0.019	0.000	0.000	0.000	0.017	0.133	0.000	0.000
	C	0.019	0.007	0.000	0.000	0.000	0.000	0.000	0.017	0.000	0.010
	D	0.000	0.000	0.000	0.000	0.000	0.045	0.100	0.000	0.078	0.010
	E	0.692	0.715	0.808	0.500	0.550	0.742	0.800	0.850	0.906	0.930
	F	0.019	0.014	0.000	0.000	0.000	0.045	0.017	0.000	0.000	0.020
	G	0.269	0.250	0.154	0.409	0.450	0.106	0.033	0.000	0.016	0.030
	H	0.000	0.000	0.000	0.000	0.000	0.045	0.000	0.000	0.000	0.000
	I	0.000	0.000	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	J	0.000	0.000	0.000	0.091	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	26	72	26	11	20	33	30	30	32	50
Pgm-2	A	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.083	0.000	0.020
	B	0.857	0.856	0.906	1.000	0.975	0.143	0.300	0.100	0.222	0.390
	C	0.119	0.122	0.094	0.000	0.025	0.857	0.683	0.800	0.704	0.580

TABLE 3. Continued.

		angustum					*asplenioides*				
Locus	Allele	Mt. Ste. Hilaire QE	Barnet VT	North Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA
	D	0.000	0.022	0.000	0.000	0.000	0.000	0.017	0.017	0.074	0.000
	E	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.010
	(N)	21	45	16	11	20	21	30	30	27	50
6Pgd-1	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	B	1.000	1.000	1.000	1.000	1.000	0.957	1.000	1.000	1.000	1.000
	C	0.000	0.000	0.000	0.000	0.000	0.043	0.000	0.000	0.000	0.000
	(N)	11	38	11	6	10	23	14	15	15	44
6Pgd-2	A	0.000	0.000	0.000	0.000	0.000	0.015	0.033	0.017	0.017	0.021
	B	1.000	1.000	1.000	1.000	1.000	0.985	0.950	0.967	0.983	0.947
	C	0.000	0.000	0.000	0.000	0.000	0.000	0.017	0.017	0.000	0.021
	D	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011
	(N)	26	69	21	11	20	33	30	30	30	47
Skdh	A	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.067	0.000	0.000
	B	0.981	0.955	0.942	0.955	1.000	1.000	1.000	0.933	1.000	0.990
	C	0.019	0.036	0.058	0.045	0.000	0.000	0.000	0.000	0.000	0.010
	D	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	26	56	26	11	20	33	30	30	32	50
Tpi-1	A	0.947	1.000	0.981	0.900	1.000	0.970	1.000	1.000	0.984	1.000
	B	0.053	0.000	0.000	0.100	0.000	0.030	0.000	0.000	0.016	0.000
	C	0.000	0.000	0.019	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	19	47	26	5	10	33	25	25	32	18
*Tpi-2**	A	0.579	0.601	0.404	0.400	0.450	0.318	0.440	0.420	0.406	0.429
	B	0.368	0.355	0.538	0.500	0.550	0.076	0.100	0.000	0.063	0.255
	C	0.053	0.036	0.058	0.100	0.000	0.606	0.460	0.580	0.531	0.316
	E	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	(N)	19	69	26	5	10	33	25	25	32	49

these eastern North American *Athyrium* populations were somewhat greater than the means for ferns (P=36.0%, A=1.65, H=0.109) obtained by averaging across 32 taxa (Li and Haufler, 1999), which are in turn similar to the means for angiosperms (P=34.2%, A=1.53, H_E=0.113; Hamrick and Godt, 1989). Mean values across *A. angustum* populations for P (34.12%) and A (1.84) were slightly lower than those for *A. asplenioides* (mean P=38.84%, mean A=2.1), while the mean value in *A. angustum* for H_E=0.138 was greater than that of 0.119 in *A. asplenioides*. This indicates that while *A. asplenioides* possesses greater allelic diversity, frequencies of alleles within loci are more evenly distributed in *A. angustum* populations.

Values for mean observed heterozygosity (H_O) were also computed for comparison to H_E. Values of H_O were very similar to (although tending to be slightly greater than) those for H_E, suggesting a tendency toward random mating.

COMPARISON TO HARDY-WEINBERG.—Genotype proportions for each polymorphic locus in each population were compared to Hardy-Weinberg expected values by computing the fixation index F, and the statistical difference of F from 0 was evaluated using the chi-square test, with pooled genotype classes if the number of alleles exceeded 2 (Table 5). The test was considered valid if two of the three genotype classes were represented by expected values ≥ 5. Of 42 validly tested loci, 40 conformed to Hardy-Weinberg expectations. Moreover, 45 out of 51 non-valid tests also indicated conformance to Hardy-Weinberg values, despite the tendency for non-valid tests to indicate false non-conformance due to low expected values. Thus, mating was inferred to approximate random mating via predominant outcrossing between gametophytes, as appears to be the general case in most ferns (Soltis *et al.*, 1988) including *Athyrium* species (Schneller, 1979).

GENETIC RELATEDNESS OF POPULATIONS AND TAXA.—The degree of genetic divergence among populations (Table 6) was quantified by computing F-statistics (Wright, 1965, 1978), including hierarchical analysis (Wright, 1978). Values for F_{ST} computed among all populations varied among loci from a low of 0.019 for *6Pgd-2* to high values of 0.468 for *Pgm-2* and 0.460 for *Idh-1*, the latter two loci having exhibited the greatest allele frequency difference between *A. angustum* and *A. asplenioides*. The value across loci of F_{ST} = 0.255 indicated very substantial differentiation among populations. This value is very high in comparison to other fern species examined, exceeding, for example, computed values for mean F_{ST} of 0.024 among populations of *Polystichum munitum* ranging from Oregon to Idaho (Soltis *et al.*, 1987), F_{ST}= 0.152 among *Pteridium aquilinum* populations ranging from Massachusetts to Florida (Speer *et al.*, 1998), and F_{ST}=0.100 to 0.248 in various species of *Dryopteris* ranging widely across eastern North America (Werth, ms.).

To evaluate the contribution of differences between *A. angustum* and *A. asplenioides* to overall population differentiation, hierarchical F-statistic analysis (Wright, 1978) was carried out (Table 6). For most individual loci, as well as for the combined values across loci, the variance between the two

TABLE 4. Estimates of genetic variation at 17 loci in ten populations of *Athyrium filix-femina s. l.* (standard errors in parentheses).

Population	Mean sample size per locus	Mean no. of alleles per locus (A)	Percent loci polymorphic[a] (P)	Mean heterozygosity	
				Observed (H_o)	Hardy-Weinberg Expected (H_E)
A. angustum					
Mt. Ste. Hilaire, QE	21.4 (1.4)	1.9 (0.2)	35.3	0.150 (0.056)	0.140 (0.047)
Barnet, VT	52.5 (3.4)	2.5 (0.3)	41.2	0.134 (0.042)	0.141 (0.042)
North Hudson, NY	21.7 (1.5)	1.8 (0.2)	41.2	0.133 (0.046)	0.136 (0.047)
Schenevus, NY	9.1 (0.8)	1.5 (0.2)	23.5	0.145 (0.071)	0.126 (0.055)
Ralston, PA	15.5 (1.4)	1.5 (0.2)	29.4	0.150 (0.061)	0.147 (0.057)
Mean	24.04	1.84	34.12	0.142	0.138
A. asplenioides					
Shirley, NJ	28.2 (2.0)	2.0 (0.3)	35.3	0.127 (0.041)	0.127 (0.040)
Mountjoy Store, VA	22.8 (2.1)	2.2 (0.3)	41.2	0.133 (0.052)	0.123 (0.042)
Hopewell, VA	23.7 (1.8)	1.9 (0.2)	47.1	0.125 (0.038)	0.118 (0.036)
Sandy Run Swamp, NC	28.9 (1.5)	2.0 (0.2)	35.3	0.104 (0.039)	0.117 (0.041)
Pond Drain, VA	44.5 (2.3)	2.4 (0.3)	35.3	0.118 (0.049)	0.112 (0.046)
Mean	29.62	2.1	38.84	0.121	0.119
Mean across all populations	26.83	1.97	36.48	0.131	0.129

[a] A locus is considered polymorphic if the frequency of the most common allele does not exceed 0.95.
[b] Unbiased estimate (Nei, 1978).

TABLE 5. Values of the fixation index F as a measure of the conformance of population genotype ratios to those expected under Hardy-Weinberg equilibrium. F was computed for each polymorphic locus in each population, and tested for statistical difference from 0 (i.e. conformance to Hardy-Weinberg expectations) using the chi-square test. Pooling was carried out for loci with more than two alleles and therefore more than three genotypic classes. Chi-square tests were considered valid only if two of the three genotypic classes were represented by an expected values ≥ 5. Results from non-valid tests are provided in brackets. Values marked as "ns" represent loci found to confirm to Hardy-Weinberg expected proportions ($p \geq 0.05$), and therefore are not statistically different from 0. Values marked with asterisks are statistically different from 0 at probabilities $p \leq 0.05$ (one asterisk), $p \leq 0.01$ (two asterisks), or $p \leq 0.001$ (three asterisks).

Locus	Mt. Ste. Hilaire QE	Barnet VT	N Hudson NY	Schenevus NY	Ralston PA	Shirley NJ	Mountjoy Store VA	Hopewell VA	Sandy Run Swamp NC	Pond Drain VA	
Ald										[−0.025 ns]	
Got		−0.040 ns				[−0.048 ns]		−0.091 ns		[−0.015 ns]	
Hk	[−0.042 ns]	[−0.028 ns]		[−0.048 ns]	[0.403 ns]	−0.124 ns	[−0.026 ns]	[−0.026 ns]	0.266 ns	[0.039 ns]	
Idh	−0.007 ns	0.102 ns	0.148 ns	[0.267 ns]	−0.099 ns	−0.208 ns	[0.653***]	−0.163 ns	0.002 ns	−0.070 ns	
Lap	[10.132 ns]	0.087 ns	0.216 ns		0.154 ns		[−0.043 ns]		[0.216***]	[−0.046 ns]	
Mdh-1	[−0.020 ns]	0.376**	[−0.061 ns]			[−0.065 ns]	[−0.034 ns]		[−0.032 ns]		
Mdh-2		[−0.010 ns]					[−0.071 ns]	[−0.053 ns]			
Mdh-3						[−0.015 ns]	[−0.034 ns]		[0.650***]		
Mdh-4	[1.000***]	0.192 ns	[−0.040 ns]				[−0.034 ns]	[−0.053 ns]			
Pgi-2	−0.375*	0.021 ns	−0.190 ns	[−0.266 ns]	−0.212 ns	−0.054 ns	0.136 ns	−0.156 ns	−0.088 ns	−0.048 ns	
Pgm-2	0.240 ns	0.120 ns	[0.103 ns]		[−0.026 ns]	−0.167 ns	−0.280 ns	0.028 ns	0.259 ns	−0.057 ns	
6Pgd-1						[1.000***]					
6Pgd-2						[−0.015 ns]	[−0.040 ns]	[−0.026 ns]	[−0.017 ns]	−0.038 ns	
Skdh	[−0.020 ns]	−0.039 ns	[−0.061 ns]	[−0.048 ns]				[−0.071 ns]		[−0.010 ns]	
No. tests showing conformance to HW[1]	3[5]	8[2]	4[5]	0[5]	3[3]	5[5]	2[7]	5[5]	5[3]	5[5]	Total: 40[45]
No. of tests showing non-conformance to HW[1]	1[1]	1[0]	0[0]	0[1]	0[0]	0[1]	0[1]	0[0]	0[2]	0[0]	Total: 2[6]

[1] number of non-valid tests in brackets.

TABLE 6. F statistic analysis (Wright, 1965, 1978), including hierarchical analysis (Wright, 1978), for 16 polymorphic loci across *Athyrium filix-femina* populations in eastern North America. Statistical difference of F_{ST} from 0 was evaluated using contingency chi-square analysis. For hierarchical analysis, *Athyrium* populations were assigned to their respective taxa (*angustum* or *asplenioides*). Differences between values of F_{ST} and $F_{locality/total}$ are attributable to differences in computational method (discussed in Swofford and Selander, 1981).

	F Statistics			Hierarchical F-statistic Analysis (variance components in parentheses)			
Locus	F_{IS}	F_{IT}	F_{ST}	$F_{Locality/Total}$	$F_{Locality/Taxon}$	$F_{Taxon/Total}$	Total Limiting Variance
Ald	−0.025	−0.002	0.022 ns	0.012 (0.00007)	0.014 (0.00009)	−0.002 (−0.00002)	0.00623
Got	−0.060	−0.017	0.041 ns	0.028 (0.00109)	0.030 (0.00116)	−0.002 (−0.00007)	0.03895
Hk	**0.116**	**0.160**	**0.049*** **	**0.021 (0.00273)**	**0.031 (0.00398)**	**−0.010 (−0.00125)**	**0.12816**
Idh-1	**0.043**	**0.485**	**0.460*** **	**0.452 (0.29124)**	**0.002 (0.00073)**	**0.451 (0.29050)**	**0.64377**
Lap	**0.077**	**0.204**	**0.137*** **	**0.113 (0.02383)**	**0.092 (0.01886)**	**0.024 (0.00497)**	**0.21012**
Mdh-1	0.036	0.060	0.025 ns	0.008 (0.00037)	0.014 (0.00068)	−0.006 (−0.00031)	0.04939
Mdh-2	−0.059	−0.013	0.044**	0.017 (0.00042)	0.016 (0.00040)	0.001 (0.00001)	0.02501
Mdh-3	0.302	0.321	0.028 ns	0.007 (0.00013)	0.002 (0.0003)	0.005 (0.00010)	0.01889
Mdh-4	0.208	0.230	0.029 ns	0.010 (0.00046)	0.014 (0.00067)	−0.005 (−0.00021)	0.04724
Pgi-2	**−0.143**	**−0.012**	**0.115*** **	**0.096 (0.03914)**	**0.040 (0.01551)**	**0.058 (0.02363)**	**0.40775**
Pgm-2	−0.001	0.469	0.468***	0.459 (0.23433)	0.045 (0.01293)	0.434 (0.22140)	0.51042
6Pgd-1	1.000	1.000	0.039 ns	0.018 (0.00016)	0.023 (0.00020)	−0.004 (−0.00004)	0.00866
6Pgd-2	−0.032	−0.013	0.019 ns	0.011 (0.00037)	0.000 (0.00000)	0.011 (0.00037)	0.03325
Skdh	−0.052	−0.019	0.031***	0.013 (0.00061)	0.011 (0.00054)	0.001 (0.00006)	0.04782
Tpi-1	−0.070	−0.020	0.047 ns	−0.003 (−0.00014)	0.000 (0.00001)	−0.004 (−0.00015)	0.04269
Tpi-2	**−0.214**	**−0.021**	**0.159*** **	**0.137 (0.08853)**	**0.013 (0.00763)**	**0.125 (0.08091)**	**0.64843**
Combined across loci	−0.052	0.217	0.255***	0.238 (0.68333)	0.028 (0.06343)	0.216 (0.601990)	

taxa with respect to the total (F_{XY}=0.216) was an order of magnitude greater than variance among populations within taxa (F_{XY}=0.028). Thus, differences between taxa explained most of the variance among populations with respect to the total (F_{XY}=0.238). This result is consistent with and explained by the large allele frequency differences at the four most polymorphic loci (*Idh-1*, *Pgm-2*, *Pgi-2*, and *Tpi-2*) between populations of different taxa as opposed to populations of the same taxon.

Values for Nei's Genetic Identity, I, (Nei, 1978) and Rogers' Genetic Similarity, S, (Rogers, 1972) were computed for each pair of populations (Tables 7 and 8). Values for these indices were consistently higher between populations of the same taxon, ranging from I=0.990 to 1.000 and S=0.930 to 0.975, than between populations of different taxa, ranging from I=0.875 to 0.938 and S=0.803 to 0.881 (Table 8).

Populations were clustered using the Unweighted Pair-group Method with Averaging (UPGMA) based on both S and I. The two indices resulted in very similar dendrograms that differed only in the association among some of the *A. asplenioides* populations; only the dendrogram based on S is illustrated (Fig. 5). Two clusters, each comprising all the populations of one taxon, were joined at S=0.849. Within the *A. angustum* cluster, the two northernmost populations Mt. Ste. Hilaire, QUE and Barnet, VT were placed as most similar, joined at S=0.975, and to this cluster the other three *A. angustum* populations were joined successively in order from north to south; the southernmost *A. angustum* population Ralston-1, PA joined the *A. angustum* cluster at S=0.938. The topology of this *A. angustum* cluster was identical in the dendrogram based on I (not shown). In *A. asplenioides*, the most similar populations based on S=0.966 were the southernmost population Sandy Run Swamp, NC and the next most southeastern occurring population Mountjoy Store, VA, and to these were joined the Pond Drain, VA population from the mountains of southwestern Virginia at S=0.958 to form a subcluster. A second subcluster, comprising the two northernmost *A. asplenioides* populations Shirley, NJ and Hopewell, VA, joined the more southern subcluster at S=0.947. The topology of the *A. asplenioides* cluster differed somewhat in the dendrogram based on I (not shown) indicating that the geographic "signal" in the *A. asplenioides* data is weaker than in the *A. angustum* data.

Discussion

The *A. filix-femina* complex, distributed across four continents and comprising as many as four North American taxa with overlapping ranges, provides an especially suitable context for exploring patterns and processes of divergent evolution and its taxonomic consequences in ferns. The two eastern North American taxa, *A. angustum* and *A. asplenioides*, long have been perceived as close relatives separable by distinctive characters that are consistent within the vast northern and southern areas they respectively occupy,

TABLE 7. Matrix of pairwise values for Rogers (1972) genetic similarity (above diagonal) and Nei (1978) unbiased genetic identity (below diagonal).

	Population									
	1	2	3	4	5	6	7	8	9	10
1. Mt. Ste. Hilaire, QE	-	0.975	0.962	0.953	0.939	0.853	0.865	0.847	0.862	0.881
2. Barnet, VT	1.000	-	0.956	0.938	0.930	0.851	0.864	0.853	0.858	0.879
3. North Hudson, NY	0.999	0.997	-	0.944	0.943	0.844	0.856	0.840	0.852	0.873
4. Schenevus, NY	1.000	0.996	0.995	-	0.942	0.841	0.842	0.831	0.843	0.863
5. Ralston, PA	0.993	0.990	0.994	0.993	-	0.817	0.821	0.803	0.825	0.843
6. Shirley, NJ	0.915	0.913	0.906	0.900	0.883	-	0.943	0.951	0.959	0.932
7. Mountjoy Store, VA	0.924	0.921	0.916	0.908	0.892	0.996	-	0.950	0.966	0.959
8. Hopewell, VA	0.912	0.911	0.903	0.893	0.875	0.998	0.997	-	0.951	0.937
9. Sandy Run Swamp, VA	0.924	0.921	0.916	0.905	0.891	0.998	1.000	0.998	-	0.956
10. Pond Drain, VA	0.938	0.936	0.934	0.923	0.911	0.990	0.998	0.990	0.996	-

but that intergrade and recombine to form a hybrid zone in their relatively narrow region of overlap. This perception is supported by the data from the present study. The spores of the two taxa show striking and consistent differences in the perispore sculpturing, a low papillate perispore in *A. angustum* versus a rugose perispore in *A. asplenioides*, and frequencies of allozymes exhibit strong differences between as compared to within the taxa. An abrupt shift in allele frequencies at the four most polymorphic loci (*Idh-1, Pgm-2, Tpi-2*, and *Pgi-2*) of *Athyrium* corresponds to the geographic boundary between *A. angustum* and *A. asplenioides*, i.e., between northern Pennsylvania and southern New Jersey in our sample. Although virtually all alleles were shared between the two taxa, some alleles that predominated or were frequent in one taxon were nearly absent in the other, e.g., $Idh\text{-}1^A$ of *A. asplenioides*, $Idh\text{-}1^C$ of *A. angustum*, and $Pgi\text{-}2^G$ of *A. angustum*. In other cases, alleles were more extensively shared between the taxa but at very different frequencies, e.g. $Idh\text{-}1^B$, $Pgm\text{-}2^B$, $Pgm\text{-}2^C$, and $Tpi\text{-}2^B$ (Table 3). UPGMA analysis of *Athyrium* resulted in two distinct taxon clusters joined at a substantially lower similarity value (S=0.849) than that joining populations within their respective taxon clusters (S=0.938 for *A. angustum*;

TABLE 8. Means of pairwise values for Rogers' Similarity (S) and Nei's Genetic Identity (I) for comparison within and between *Athyrium* taxa. Ranges are given in parentheses. Each category was represented by ten pairwise comparisons.

Taxon combination	I	S
angustum–angustum	0.996 (0.990–1.000)	0.948 (0.930–0.975)
asplenioides–asplenioides	0.996 (0.990–1.000)	0.951 (0.932–0.966)
angustum–asplenioides	0.911 (0.875–0.938)	0.848 (0.803–0.881)

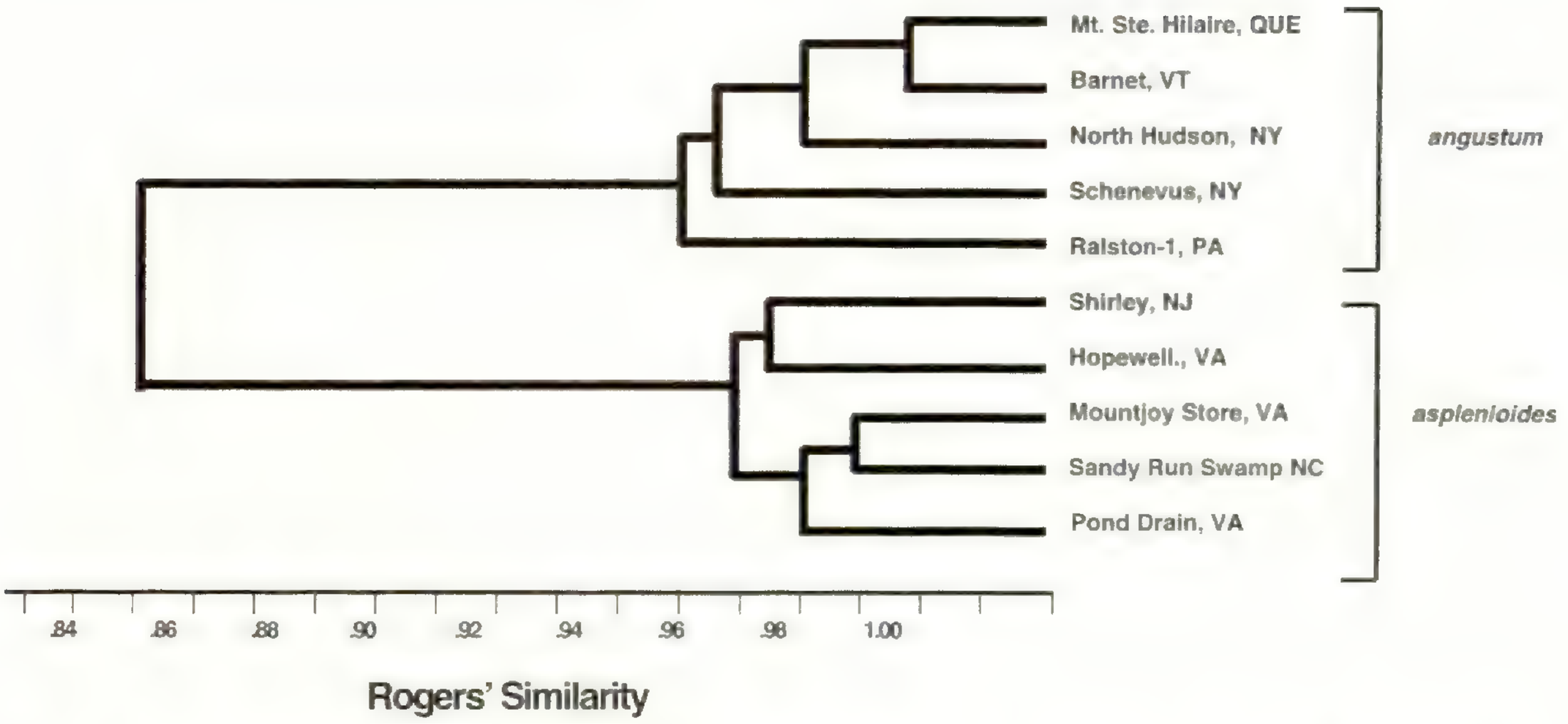

FIG. 5. Dendrogram resulting from UPGMA analysis based on pairwise values of Rogers' Similarity (Table 7).

S=0.947, for *A. asplenioides*). These spore and allozyme differences for the most part are consistent among populations of each taxon, yet there is evidence of introgressive hybridization in their region of overlap as discussed below.

The distinctness of these taxa and the consistency of the character differences specified by Butters (1917) frequently have been questioned by statements indicating that the taxa intergrade extensively (e.g., Benedict, 1934; Weatherby, 1936; Fernald, 1946; Shaver, 1954; Wherry, 1961). However, such statements seem anecdotal in that they are not accompanied by documentation of character combinations in specimens. The most extensive specimen-based data set is that of Liew (1971, 1972), who carried out a phenetic analysis of North American *Athyrium sensu lato* based on 170 specimens scored for 99 characters. Liew indicated that cluster analysis based on paired similarity coefficients separated each *Athyrium* taxon, including *A. angustum* and *A. asplenioides*, into its own cluster, but the summary dendrograms presented leave it uncertain as to whether some of the specimen placements were inappropriate or equivocal.

In the present study, the degree of differentiation between *A. angustum* and *A. asplenioides* may appear exaggerated by the omission of localities where the two taxa co-occur. This omission was neither intentional nor an oversight, rather it resulted from a failure to discover such localities despite a substantial effort to do so. A search for co-occurring populations in southern Pennsylvania resulted in finding only a few isolated individuals of *A. asplenioides* in this highly agriculturalized and urbanized region, and no sizable populations from which allele frequency data could be obtained. All individuals in populations sampled for the present investigation from Quebec south to northern Pennsylvania were readily assignable to *A. angustum* on the basis of leaf and spore morphology. Similarly, all individuals in

populations from North Carolina north to northern Virginia were assignable to *A. asplenioides*. However, a genetic influence from *A. angustum* is suggested by the high frequency of characteristically *A. angustum* alleles $Idh\text{-}1^B$ and $Pgi\text{-}2^G$ in the northernmost *A. asplenioides* population Shirley, NJ, as well as $Pgm\text{-}2^B$ and $Tpi\text{-}2^B$ in the highest elevation *A. asplenioides* population Pond Drain, VA. Evidence of introgressive hybridization is augmented by the intermediate spore morphologies encountered at the Shirley, NJ, locality. No individuals assignable to *A. angustum* were found at this site, but it is possible that *A. angustum* spores could have migrated from populations further north and effected hybridization (Wagner, 1943). The prevalence of fully intermediate spores in six of the individuals, five of which are heterozygous for taxon marker allozymes, suggest that these are first-generation hybrids. The skewed array of spore morphologies of other individuals suggests that they are backcrosses and provides evidence that hybridization has gone beyond the first generation resulting in introgression. Thus, spore and isozyme data combine to support previous morphology-based suggestions that the region of overlap between the taxa, and possibly higher elevations in the southern Appalachians as well, could represent a hybrid zone resulting from secondary contact between recently diverged sister taxa (Benedict, 1934; Shaver, 1954; Sciarretta *et al.*, ms).

Narrow hybrid zones in which formation of fertile hybrids and backcrosses result in intergradation between divergent taxa occur between numerous species or subspecies pairs of animals and angiosperms (reviewed by Arnold, 1997), and occur in a few agamosporous ferns (Gastony and Windham, 1989). Hybridization between fern species usually results in spore abortion due to abnormal meiosis, and introgressive hybridization between homoploid taxa as divergent as *A. angustum* and *A. asplenioides* is decidedly rare in ferns, with only three cases having been documented previously: (1) swarms of fully fertile hybrids involving *Pteris quadriaurita* and *P. multiaurita*, first generation as well as backcrosses, occur in disturbed forests of Sri-Lanka (Walker, 1958); (2) extensive hybridization among three species of the tree fern genus *Alsophila* resulted in a complex swarm of fertile hybrids in Puerto Rico, a scenario hypothesized to give rise to new species through allogamous allohomoploidy (Conant and Cooper-Driver, 1980; Conant, 1990); (3) morphological and molecular data provide evidence of hybrid swarms between *Polystichum munitum* and *P. imbricans* of northwestern North America (Mayer and Mesler, 1993; Mulleniex *et al.*, 1998). Of these, the situation in *Polystichum* most closely parallels that of the *Athyrium* taxa *angustum* and *asplenioides*. The two *Polystichum* taxa share alleles at most enzyme loci, but are differentiated with respect to frequencies at three loci resulting in a lower magnitude of genetic identity between the taxa (I=0.842) than within each taxon (means: I=0.974 for *P. imbricans*, I=0.957 for *P. munitum*; Soltis *et al.*, 1990, 1991). Additionally, the *Polystichum* taxa hybridize introgressively, although they do not form a hybrid zone as in *Athyrium*; rather hybridization occurs at various localities across a broad area of sympatry between the two taxa (Mayer and Mesler, 1993; Mulleniex *et al.*,

1998). Maintenance of distinction between these two *Polystichum* species most likely results from a combination of their partial intersterility and diversifying selection imposed by the very different habitats—forests versus exposed cliffs—occupied by *P. munitum* and *P. imbricans* respectively.

The nature, frequency, and geographic extent of hybridization between *A. angustum* and *A. asplenioides* remain uncertain and merit further research that combines field, herbarium, molecular, and breeding studies. It is critical to determine with greater precision and full documentation the degree to which these two taxa maintain versus blend their macromorphological, micromorphological, and allozymic differences where they coexist. It is unknown whether there is preferential mating within taxa, whether taxon characters tend to remain associated in the face of hybridization or are completely recombined, and whether there exists a cline for allozyme frequencies and morphological characters within the overlap region. There is a need for intensive exploration for coexisting *A. angustum* and *A. asplenioides* populations in the areas of their overlap from the eastern seaboard through the midwest, and in the southern Appalachians where the existence of cryptic taxa has been hypothesized (Wagner and Wagner, 1966). Isozyme studies of these populations should be coordinated with critical analyses of morphological character combinations obtained from numerous specimens and with experimental crosses that can quantify the propensity of the taxa to hybridize.

Beyond the taxonomic significance of hybridization, the unprecedented formation of normal intermediates between spores of such divergent morphology provides an opportunity to gain insight into the genetics underlying spore morphology (Schneller, 1989). The variability of spore morphology within individuals of the putative primary hybrids from the Shirley, NJ site, which includes expression of parental types, indicates that inheritance is polygenic rather than a simple one-or-two-gene inheritance mechanism, and that the spore genotype determines or at least influences perispore phenotype. Contrasting observations and inferences were obtained by Schneller (1989), who reported the formation of normal intermediate spores in experimentally produced hybrids between *A. angustum* and *A. asplenioides*, but found that all spores from a sporangium were of the same type, implicating sporophytic determination of the perispore morphology. Explanations that can account for these differing observations include the possibility that the Shirley, NJ, plants were not true F1 hybrids, or that there may be a maternal effect that varies as a polymorphism. Clearly, further studies of the inheritance of spore morphology are in order.

TAXONOMIC CONCLUSION: AT WHAT RANK SHOULD *A. ANGUSTUM* AND *A. ASPLENIOIDES* BE RECOGNIZED?—Over the second half of this century, the nature of pteridophyte species has been clarified significantly by application of technological advances in cytology and molecular systematics in combination with detailed morphological and field studies (Manton, 1950; Wagner, 1963; Haufler, 1987, 1989; Paris *et al.*, 1989; Conant, 1990). Nonetheless, definitive ranking of closely related divergent taxa with overlapping ranges, such as

the two *Athyrium* taxa considered here, remains challenging due to the unsettled controversy as to the nature and definition of species (e.g., Mayr, 1992; Davis and Nixon, 1997; Baum, 1998; DeQueroz, 1998) as well as to the unpredictable nature of reproductive interactions between diverged taxa experiencing secondary contact (Arnold, 1997). The rank assigned to *A. angustum* and *A. asplenioides* has varied considerably in floristic treatments published in this century. While Small (1938) and Wherry (1948, 1961) followed Butters (1917) in treating these taxa as separate species, other authors have tended to treat them as infraspecific taxa, either subspecies (Lellinger, 1985) or varieties (Fernald, 1950; Mickel, 1979; Cody and Britton, 1989; Gleason and Cronquist, 1991; Kato, 1993).

Spore and isozyme data combined indicate that populations of these two taxa are significantly divergent, exhibiting greater differences than ordinarily encountered within single species of ferns thus far studied. However, the fertility of hybrids (Schneller, 1989) provides a potential for introgression between the two taxa. The preliminary evidence that hybridization and introgression do in fact occur indicates that *A. angustum* and *A. asplenioides* would be considered conspecific under species definitions as different as the Biological Species Concept (Mayr, 1942) and the Phylogenetic/Diagnostic Species Concept (Cracraft, 1983; Davis and Nixon, 1992). Nonetheless, in practice numerous pairs of taxa that form hybrid swarms or zones are treated as distinct species (Grant, 1981; Arnold, 1997).

The occurrence of northern and southern infraspecific taxa in eastern North American *Athyrium* is paralleled in *Pteridium aquilinum* L., which comprises northern and southern varieties *latiusculum* (Desv.) Underw. and *pseudocaudatum* (Clute) Heller, respectively, and which shows north to south clines in allele frequencies near the overlapping taxon boundary (Speer *et al.*, 1998). However, the pattern of allozyme variation in *Pteridium* differs from that in *Athyrium* in that the most abrupt shift in allele frequencies occurs within the range of *P. latiusculum*, genetic identities between populations of the two *Pteridium* varieties ranged substantially higher (I= 0.916 to 0.999) than those of the two *Athyrium* taxa (I=0.875—0.938, mean =0.911), and UPGMA clustering failed to separate the varieties (Speer *et al.*, 1998). On the basis of the lack of genetic differentiation between *P. latiusculum* and *P. pseudocaudatum*, variety was indicated as the highest rank at which to recognize them (Speer *et al.*, 1998). In contrast, the more substantial differentiation between the *Athyrium* taxa *angustum* and *asplenioides* and the consistent characters uniting a vast number of individuals north and south of their hybrid zone suggest that the taxa should be ranked at least at the level of subspecies. Ranking at the species level would not be inconsistent with the treatment of such taxa in the broader plant literature.

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A New Hybrid *Polypodium* Provides Insights Concerning the Systematics of *Polypodium scouleri* and its Sympatric Congeners

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Abstract.—With its thick, leathery leaves, reticulate venation, and large sori, *Polypodium scouleri*, located in a narrow band along the Pacific coast of North America, is the most distinctive member of the cosmopolitan *P. vulgare* species complex. Although early studies based on morphology and chromosomes yielded hypotheses about the relationships among some elements of this complex, phylogenetic alliances to *P. scouleri* were not proposed. Combining data from *rbcL* and *trnL* DNA sequences with isozymic analyses suggested that *P. scouleri* originated relatively recently and is closely allied to and sympatric with *P. californicum* and *P. glycyrrhiza*. Consistent with a hypothesis of recent origin, we detected no infraspecific isozymic variation across the range of *P. scouleri*. Although allopolyploidy is a common feature of the *P. vulgare* complex, *P. scouleri* stands out because it has not been implicated in the origin of any secondary (allotetraploid) species. However, as early as 1951, Manton reported a triploid individual that was morphologically similar to *P. scouleri*, but whose other parent could not be verified. Since that time, others have suggested that *P. scouleri* might be crossing with sympatric congeners, but no solid evidence has been obtained. The present study confirmed that *P. scouleri* hybridized with neighboring *P. calirhiza*, and showed that individuals with intermediate morphological features contained isozyme marker alleles from both parental lineages.

The *Polypodium vulgare* L. complex (Polypodiaceae) first drew attention with the publication of cytological counts for several European and North American members (Manton, 1950). Controlled crosses among members of the complex followed (Shivas, 1961), and resulted in further taxonomic studies founded on interbreeding boundaries and subtle morphological distinctions. A major organizational leap in the circumscription of North American *Polypodium* L. species was the definition of eastern and western complexes (Lloyd and Lang, 1964). In their search to identify parental lineages for allopolyploid members, Lloyd and Lang grouped the complexes on the presence (eastern) or absence (western) of sporangiasters. Although sporangiasters were later shown to constitute synapomorphies in several western species (*P. amorphum* Suksdorf, *P. saximontanum* Windham, and *P. sibiricum* Siplivinsky [Haufler and Windham, 1991]), recognizing the importance of these unique soral features within American polypodiums was insightful. Additionally, Lloyd and Lang (1964) hypothesized that the progenitors of the allotetraploid *P. californicum* Kaulfuss were *P. glycyrrhiza* D. C. Eaton (2*n*)

and diploid *P. californicum* Kaulfuss. It was not until much later, however, that Whitmore and Smith (1991) described the tetraploid cytotype as a distinct and reproductively competent species, *P. calirhiza* S. A. Whitmore & A. R. Smith.

Research explorations of morphology (Haufler and Windham, 1991; Whitmore and Smith, 1991; Haufler *et al.*, 1993), cytology (Haufler and Wang, 1991), isozyme variation (Haufler *et al.*, 1995b), chloroplast DNA restriction site analysis (Haufler *et al.*, 1995a), and DNA sequence data (Haufler and Ranker, 1995) have further defined relationships within the *Polypodium vulgare* complex. Yet a review of the above reveals the exclusion of *P. scouleri* Hooker & Greville from all but two studies (Haufler *et al.*, 1993; Haufler and Ranker, 1995).

Confined to a narrow distribution along the Pacific Coast of North America, and tentatively recognized as a member of the western complex, the distinctive morphology of *P. scouleri* clearly separating it from its congeners precluded the formulation of accurate hypotheses about phylogenetic relationships. The overview by Haufler *et al.* (1993, pp. 315–323) in their treatment of *Polypodium* in Flora of North America (Fig. 1) allied *P. scouleri* with other western members, and provided a detailed morphological description. In addition, the investigation of *rbcL* sequence data suggested a sister taxon relationship with *P. glycyrrhiza* that was supported by isozyme profiles (Haufler and Ranker, 1995). Haufler and Ranker (1995) hypothesized that the particularly distinctive morphological features of *P. scouleri* may have evolved through adaptive response to environmental stress. That study did not consider another close relative, *P. californicum*, and, because only diploids were included, did not incorporate allopolyploid *P. calirhiza*.

Recently, leaves resembling *P. scouleri* but having some atypical features were collected at a site in California. At the same time, leaves of *P. calirhiza* and more typical *P. scouleri* were obtained. The present study was designed to examine the atypical leaves morphologically and use molecular approaches to determine whether these plants originated through hybridization between *P. scouleri* and other members of the western complex. Several morphological features of suspected hybrid leaves were investigated and compared with features of typical *P. scouleri* and *P. calirhiza* leaves. Starch gel electrophoresis was used to reveal isozyme marker alleles and characterize the suspected hybrid and parental lineages.

Material and Methods

Study Area.—Located within the confines of highly populated San Francisco County are the natural vegetation preserves of Tank Hill and Mt. Sutro (Fig. 2). The Open Space Program of the San Francisco Recreation and Parks Department retains ownership of these sanctuaries. Land stewards manage vegetation of the preserves, emphasizing enhancement and restoration of native species as well as the removal of invasive exotic plant populations.

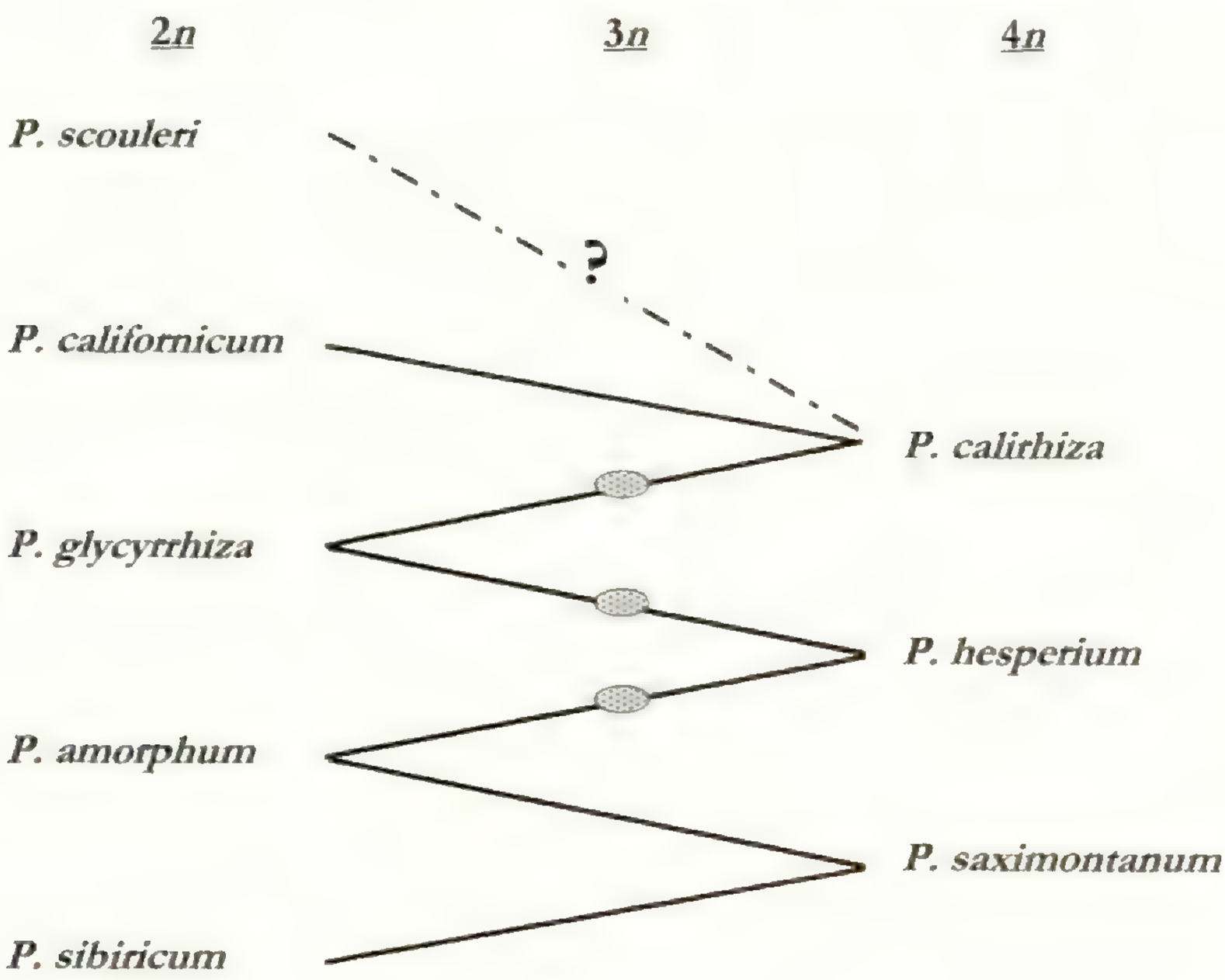

FIG. 1. Kinship of diploid and tetraploid members of the Western *Polypodium vulgare* complex from Haufler, *et al.* (1993). All taxa are restricted to western North America with the exception of *P. sibiricum*, which is circumboreally distributed in North America and Asia. Shaded ovals represent sterile backcrosses (3*n*) in the complex. The dashed line between *P. scouleri* and *P. calirhiza* indicates the subject of the present study.

A dense forest of mature, non-native cypress (*Cupressus macrocarpa* Hartw. Ex Gord.) and eucalyptus (*Eucalyptus globulus* Labill.) was planted on Mt. Sutro as early as 1870 and now covers much of the hill. *Polypodium* species on Mt. Sutro flourish beneath the forest canopy, nestled among rocks and at the bases of trees as hemiepiphytes. Leaves of *Polypodium calirhiza* and *P. scouleri* were collected from the east face of Mt. Sutro (Site 1, Fig. 2; Table 1).

Eucalyptus and cypress were also planted on Tank Hill, but, in contrast to Mt. Sutro, they are sporadic on Tank Hill, and primarily at lower elevations. The summit of Tank Hill is dominated by large tracts of rocky fields and ledges of Franciscan radiolarian chert. In the fields and on the ledges are *Polypodium* populations exposed to the harsh sun and buffeted by gusting winds. Native species associated with ferns in this predominantly open habitat include: Nootka reed grass (*Calamagrostis nutkaensis* (Presl) Steud.), yarrow (*Achillea millefolium* L.), coast barberry (*Berberis pinnata* Lag.), soap plant (*Chlorogalum pomeridianum* (DC.) Kunth. var. *divaricatum* (Lindl.) Hoov.), and seaside daisy (*Erigeron glaucus* Ker.). Among the boulders and crevices near the crest of Tank Hill, leaves of *Polypodium calirhiza* and

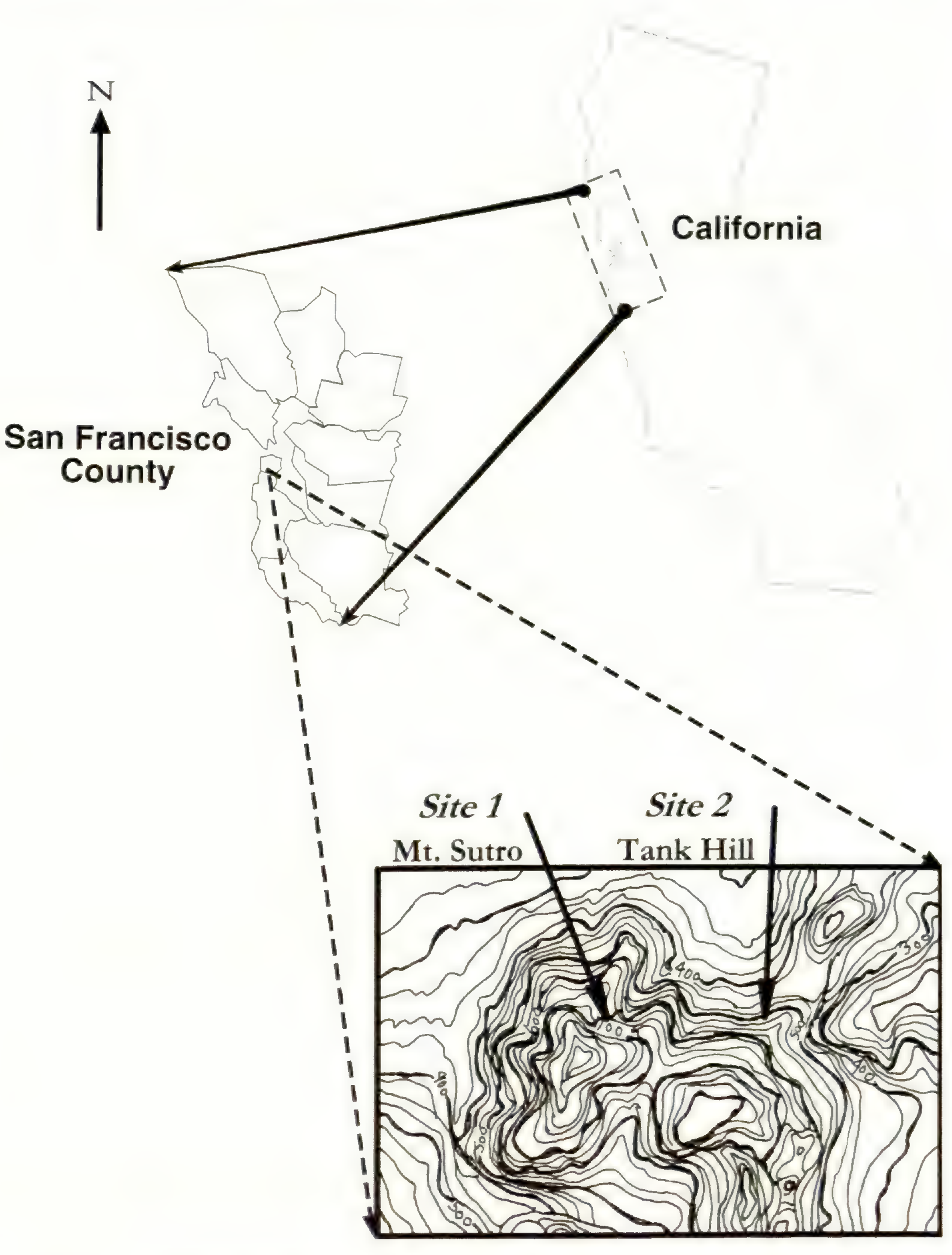

FIG. 2. Collection localities on Mt. Sutro and Tank Hill, San Francisco County, California.

P. scouleri, as well as the suspected hybrid, were collected (Site 2, Fig. 2; Table 1). To characterize the amount of electrophoretically detectable genetic variation across the range of *P. scouleri*, population samples were obtained from four additional California populations. Representative specimens of all

TABLE 1. California sites from which *Polypodium* plants were collected. All specimens are deposited in the McGregor Herbarium, University of Kansas (KANU). N = number of individuals for each population.

Collection Site (county)	Species (ploidy level: x = 37)	N	Voucher
Mt. Sutro (San Francisco)	*P. calirhiza* (4x)	5	*Hildebrand 3217*
Mt. Sutro (San Francisco)	*P. scouleri* (2x)	3	*Hildebrand 3216*
Tank Hill (San Francisco)	*P. calirhiza* (4x)	12	*Hildebrand 3219*
Tank Hill (San Francisco)	*P. scouleri* (2x)	7	*Hildebrand 3218*
Tank Hill (San Francisco)	*P. calirhiza* × *scouleri* (?)	5	*Hildebrand 3220 & 3221*
Fern Canyon (Trinity)	*P. scouleri* (2x)	20	*Therrien s.n.*
Point Reyes National Seashore (Marin)	*P. scouleri* (2x)	15	*Therrien s.n.*
Trinidad (Humboldt)	*P. scouleri* (2x)	6	*Therrien s.n.*
Fort Ross (Sonoma)	*P. scouleri* (2x)	3	*Therrien s.n.*

collections (Table 1) were pressed and deposited at McGregor Herbarium of the University of Kansas (KANU).

METHODS.—Leaves were removed from live material in the field, placed in plastic bags, and shipped on ice. Upon arrival, bags were transferred to 4°C storage where they were kept until preparation for electrophoresis. At least one sample of plant material for each leaf was prepared immediately upon receipt in the laboratory. Most leaves, particularly of *P. scouleri* and the suspected hybrid, retained their fresh appearance in storage for considerable time (up to one month). Preparations from fresh plant material stored for longer periods yielded banding patterns comparable to that prepared immediately, indicating extended retention of enzymatic activity.

Plant material was prepared by crushing in phosphate-PVP buffer (Soltis *et al.*, 1983) followed by absorption of the homogenate into filter paper wicks and storage of the wicks at −80° C. Freezing prepared fresh material allowed storage of samples for several months with no loss of enzymatic activity.

Selection of enzymes and the systems best suited for their survey was based on previous studies of *Polypodium* (Haufler *et al.*, 1995a, 1995b) and other fern genera (Haufler, 1985b; Haufler *et al.*, 1990; Soltis *et al.*, 1990; Pryer and Haufler, 1993). Banding patterns were obtained by electrophoresis on 12.4% starch gels for the following enzymes: aldolase (ALD), fructose 1,6-bisphosphatase (FBP), glyceraldehyde 3-phosphate dehydrogenase (G-3PDH), hexokinase (HK), isocitrate dehydrogenase (IDH), phosphogluconate dehydrogenase (PGDH), triosephosphate isomerase (TPI), and phosphoglucoisomerase (PGI). Bands were resolved best for ALD and IDH on system 11 (Haufler, 1985b) whereas only the 7.5 pH version of the morpholine/citrate system (MC) (Clayton and Tretiak, 1972) revealed clear bands for G-3PDH. Both system 11 and MC resolved bands for FBP, MC and system 8 (Haufler, 1985a) for MDH, and MC and system 6 (Soltis *et al.*, 1983) for PGDH. System 8 also revealed bands for HK, LAP, and, in addition to system 6, for PGI. TPI bands were revealed only with system 6. Digital images were obtained of all

TABLE 2. Comparisons of character states identified for *Polypodium scouleri*, *calirhiza* × *scouleri*, and *P. calrhiza*.

Character	*Polypodium scouleri*	*Polypodium calirhiza* × *scouleri*	*Polypodium calirhiza*
Frond texture	stiff, leathery	stiff, leathery	herbaceous
Rhacis scales	broadly ovate, tapering to ca 3 cells in width; not occurring in pairs	narrowly ovate to broadly lanceolate; often adjacent and fused (from the base) to one third their length	lanceolate to lanceolate-ovate; 3–6 cells wide, tapering to 1–3 cells; not occurring in pairs
Pinna venation	regularly anastomosing, forming one row of areoles	mixed, primarily free but occasionally anastomosing and forming areoles	free, no areoles formed
Guard cells			
- shape	round	round	elliptic
- mean length: μm (range)	35 (33–38)	38 (35–43)	56 (48–63)
Subsidiary cell margins	smooth	distinctly lobed	distinctly lobed
Approximate stomata density	150/mm^2	70/mm^2	40/mm^2
Spore length: μm (s.d.)	61.3 (± 8.4)	malformed	61.5 (± 7.1)
Sori			
- shape	round or oblong; shape specific to individual plant	round or oblong; both shapes on individual plants	oblong
- distribution	closest to midrib	variable	midway between costa and margin
- size	generally > 3 mm	variable, 1–4 mm, to absent	generally < 3 mm

gels using a Nikon CoolPix 950 camera and visualized with Adobe Photoshop 5.5 software for Macintosh.

Morphological characters documented as useful for delimiting *Polypodium* species (Haufler *et al.*, 1993; Whitmore and Smith, 1991) were examined on leaves from both parental species and the hybrid. These included leaf texture, sorus diameter, pinna venation, spore length, and rachis scale width. In addition, lower surface (abaxial) epidermal peels were produced to measure sizes and characterize shapes of guard and subsidiary cells, as well as differences in stomatal density (Table 2).

RESULTS

MORPHOLOGY.—The leaves of *Polypodium scouleri* differ from those of other members of the genus (although leaves of *P. calirhiza* may become somewhat thickened in exposed coastal environments) by their leathery texture, and

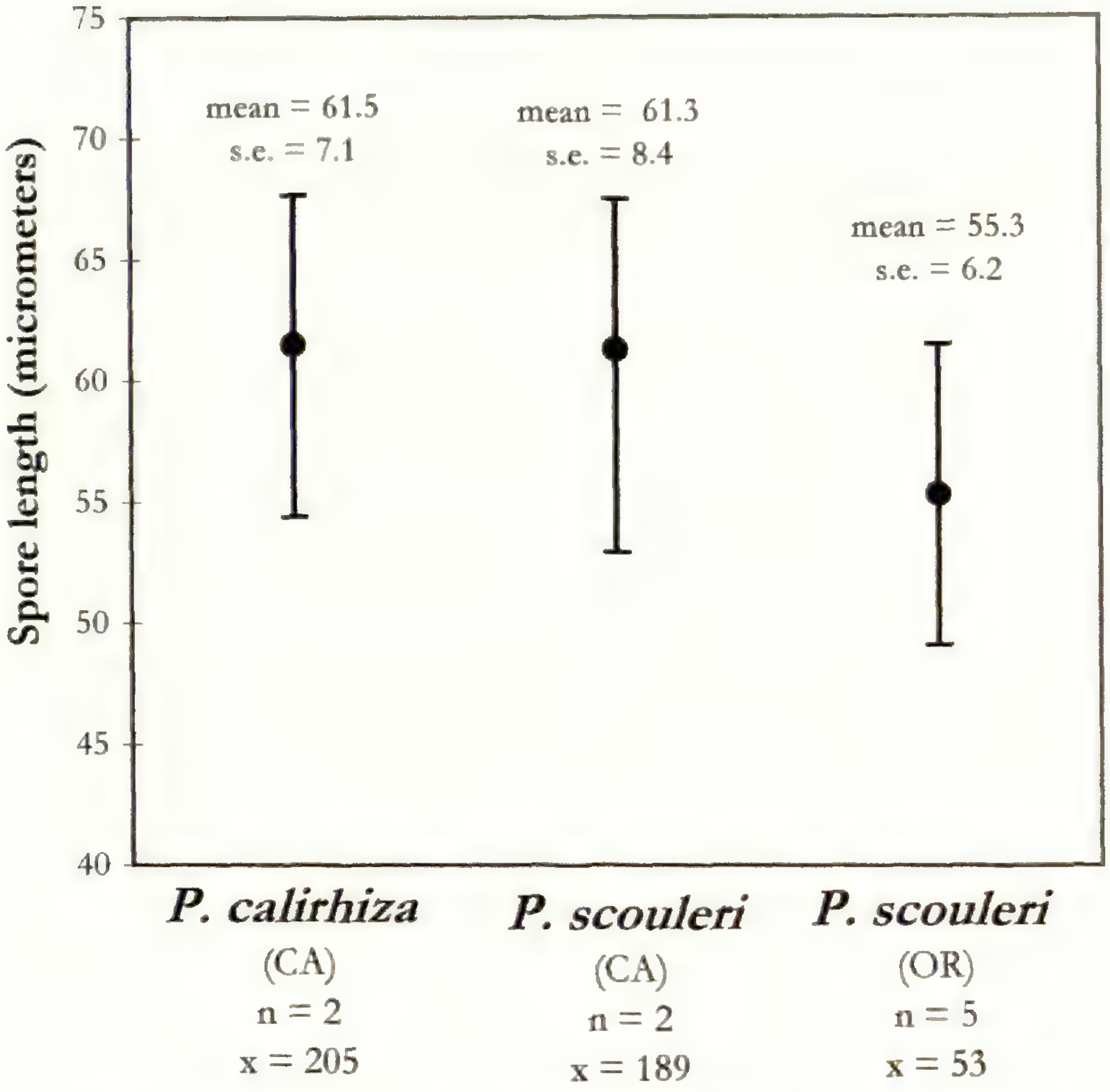

FIG. 3. Comparisons of spore length from plants gathered at the Mt. Sutro and Tank Hill *Polypodium* populations, and from a *P. scouleri* population from Oregon. Vertical bars represent +/− one standard error. Expected mean spore lengths (Haufler *et al.*, 1993) for *P. calirhiza* and *P. scouleri* are > 58 μm and < 53 μm, respectively. n = number of plants sampled; x = number of spores measured.

their well-defined aeroles produced by anastomosing venation. Other distinctive morphological features include individual leaf segments that are greater than 12 mm in width, soral diameters of more than 3 mm, and rachis scales that are large, pale reddish brown and broadly triangular, tapering to a point less than three cells wide (Table 2). The pair of guard cells surrounding the stomata on the lower epidermal surface is circular in outline; individual guard cells average 35 μm in length, and are adjoined by smooth-margined subsidiary cells. Stomata density is approximately 150 stomata/μm^2. Tank Hill and Mt. Sutro *P. scouleri* populations had a mean spore length of 61.3 μm (Fig. 3).

Polypodium calirhiza plants have herbaceous leaves lacking the leathery texture of *P. scouleri* and are, in general, of smaller stature. Pinna venation is free or weakly anastomosing with some to many segments lacking aeroles. Leaf segments seldom exceed 12 mm wide, and soral diameters are less than 3 mm. Rachis scales, as in *P. scouleri*, are a translucent, pale reddish brown,

but, in contrast to *P. scouleri*, are lanceolate, only a few cells wide proximally, and narrow to only one to three cells distally. Epidermal peels showed elliptic paired guard cells (average length: 56 μm) and subsidiary cells with distinctly lobed margins. In striking contrast to that observed for *P. scouleri* stomata, stomata density is approximately 40 stomata/mm^2. Spores of the California *P. calirhiza* plants averaged a mean length of 61.5 μm.

The putative hybrid individuals showed both hybrid vigor (Charlesworth and Charlesworth, 1987) and dwarfing of leaves. On nearby Mt. Davidson, a more sheltered locale, *P. scouleri* produced leaves greater than 70 cm in length and lush in appearance. *Polypodium calirhiza* morphotypes vary with exposure, and increasingly open areas produce plants of more diminutive stature.

Morphological character states observed for hybrid leaves were either i) a combination of discrete features inherited without change from each parent, or ii) an additive blending of parental traits resulting in intermediate morphological states (Table 2). An exception was the consistently leathery, stiff texture of hybrid leaves, a phenotype that resembles only the *P. scouleri* parent. Leaves of the hybrid never had the herbaceous texture of the *P. calirhiza* lineage.

Pinna venation of hybrid leaves incorporates features of both parents: most veins are free, but occasional anastomoses and areoles occur. Soral size and development are extremely variable on hybrid leaves and of three general categories. Sori were 1) as large or slightly larger than those typical of *P. scouleri* (> 3 mm), 2) smaller and resembling *P. calirhiza* sori (< 3 mm), or 3) entirely undeveloped. Translucent, reddish brown scales occur along the rachis of hybrid leaves, but, in contrast to the deltate and lanceolate parental scales (*P. scouleri* and *P. calirhiza*, respectively), rachis scales on hybrid leaves are narrowly triangular. In addition, adjacent rachis scales are often fused (from the base) for approximately one third their length. Paired guard cells were orbicular in outline, as observed for *P. scouleri*, whereas subsidiary cells showed the distinctive, lobed margins of *P. calirhiza*. The average length of guard cells (38 μm), and stomatal densities of approximately 70 stomata/mm^2 are intermediate between parental lineages. All spores on hybrid plants were shrunken and malformed, suggesting inviability.

MOLECULAR.—Previous isozymic work by Haufler *et al.* (1995b) investigated *P. californicum* and *P. glycyrrhiza* (the progenitors of *P. calirhiza*) and identified electrophoretic markers for each diploid member, based on sampling the infraspecific variation from eleven populations. In the present study, isozyme profiles of the Tank Hill and Mt. Sutro individuals of *P. scouleri* were verified as representative for the species by sampling other populations (Table 1). In contrast to the genetic variability detected for other *Polypodium* diploids, *P. scouleri* isozymes were monomorphic across all populations and for all enzymes sampled. Of the ten enzyme systems considered, four (HK, PGI, PGDH, and MDH) yielded reproducible, well resolved banding patterns

that discriminated individuals representing parental species and hybrids. Allozymes (allelic variants within loci) are distinguished from isozymes (products from different loci) by assuming that models of gene product compartmentalization (Gastony and Darrow, 1983) are applicable to *Polypodium* enzymes.

The following results were obtained for each of the applicable enzyme systems. Gels stained for the monomeric enzyme hexokinase (HK) showed a slow-migrating allele in *P. calirhiza* samples in contrast to the faster allele present in *P. scouleri*. The hybrid expressed both alleles, one from each parental species (Fig. 4a). Two isozyme loci were resolved for phosphoglucoisomerase (PGI) and phosphogluconate dehydrogenase (PGDH). The faster migrating isozymes (*Pgi* 1, *Pgdh* 1) for both enzymes were monomorphic for all samples investigated. In contrast, banding patterns for the slower migrating isozymes (*Pgi* 2, *Pgdh* 2) were more variable for each enzyme (Fig. 4b & 4d). *Polypodium calirhiza* possessed a slower migrating allozyme for *Pgi* 2, whereas *P. scouleri* revealed a faster allozyme. The hybrid exhibited an additive banding pattern for dimeric phosphoglucoisomerase, possessing both allelic variants (Fig. 4b). A similar pattern was observed from gels stained for phosphogluconate dehydrogenase (PGDH). The polymorphic locus (*Pgdh* 2) expressed only the faster migrating allele in *P. calirhiza*, both allelic variants in *P. scouleri*, and an additive banding pattern in the hybrid (Fig. 4d). Three isozymes were revealed for malate dehydrogenase of which two (*Mdh* 2, *Mdh* 3) were monomorphic across all samples. The polymorphic locus (*Mdh* 1) produced bands that represent a fast allele for *P. calirhiza* and a slower migrating allele present in *P. scouleri* samples. In addition, both intra- and inter-locus heterodimers were formed during electrophoresis and were subsequently revealed by staining for malate dehydrogenase. Bands produced by the formation of both intra- and inter-locus heterodimers further supported the hybrid pattern of additive banding from parental species (Fig. 4c).

Discussion

Hybridization between species is a frequent phenomenon in plants and is especially common in pteridophytes (Wagner, 1968). The clarity and precision of species recognition and the accuracy of phylogenetic hypotheses can be enhanced by identifying and characterizing naturally occurring hybrids. Especially in groups such as the *Polypodium vulgare* complex, where species differences are particularly subtle, unrecognized interspecific hybrids that usually blend features of the parental individuals can appear to bridge gaps between otherwise distinct species.

Zymograms were exceptionally informative for delimiting *P. scouleri* and *P. calirhiza*, and for unequivocal verification of hybrid leaves collected on Tank Hill and Mt. Sutro. Typically, additive banding patterns indicate the presence of both parental alleles and are observed in hybrids (Crawford, 1990; Murphy *et al.*, 1996). The additive banding patterns visualized on gels

FIG. 4. Representative gels stained for enzymes from *Polypodium* samples included in the present study. *P. calirhiza* = ca; *P. calirhiza* × *scouleri* = casc; *P. scouleri* = sc. A. hexokinase (HK); B. phosphoglucoisomerase (PGI); C. malate dehydrogenase (MDH); and D. phosphogluconate dehydrogenase (PGDH). Sampling in B. & C. corresponds to species as labeled in D. See text for interpretation of banding patterns.

stained for enzymes HK, PGI, PGDH, and MDH combine and confirm parental contributions from the *P. scouleri* and *P. calirhiza* genomes to the hybrid.

The difficulty encountered in developing equivalently distinctive morphological characterizations of the hybrid individuals requires further discussion. Hybrids are often recognized initially because they have morphological peculiarities that can signal the amalgamation of two distinct genomes. Wagner (1962) reviewed deviations from morphological symmetry and their role as indicators of hybrid origins in ferns. For genera studied (*Asplenium*, *Cystopteris*, *Cheilanthes*, *Osmunda*, *Polystichum*, *Pteris*, *Woodsia*), he developed three broad conclusions regarding hybrid morphology: First, hybrid structures form symmetrically, but blend traits from parental lineages. If large differences occur between parental lineages, hybrids tend toward irregular or asymmetric development. Second, when asymmetric development does occur, it is retained in hybrids, and may be useful in identifying the hybrid individual. Third, the discovery of morphological irregularities should key investigators to the possibility of hybrid origins and further study of possible parental lineages. Thirty years of further investigation of these fern genera (e.g., Moran, 1982; Murakami *et al.*, 1999; Yatskievych *et al.*, 1988; Mickel, 1979; Haufler *et al.*, 1990), in addition to other pteridophytes (e.g., Montgomery, 1982; Palmer, 1998; Pryer and Haufler, 1993; Tyron, 1968) support the conclusions on hybrid morphology summarized by Wagner. Likewise, our study reported intermediate, blended traits in *P. calirhiza* × *scouleri* that are consistently expressed in all leaves, and that help to resolve the definition of the hybrid and the identification of parental species. In comparison, other character states were not intermediate between parental lineages, or were so highly variable that they could not contribute to the definition of *P. calirhiza* × *scouleri* (Table 2).

The loss of reticulate venation found in *Asplenium* and *Polystichum* (Wagner, 1962), and other *Polypodium* hybrids (Whitmore and Smith, 1991) is also observed in *P. calirhiza* × *scouleri*. Whitmore and Smith (1991) investigated other members of the western *P. vulgare* complex, and revealed a loss of vein anastomosis following hybridization. They observed only 0–33% of the veins per pinna in *Polypodium calirhiza* anastomose, whereas parental species *P. californicum* has weakly to fully anastomosing venation (5–100%) and *P. glycyrrhiza* produces pinna with entirely free venation. The primarily free venation occurring in *P. calirhiza* × *scouleri* provides further evidence for a propensity toward less reticulated venation whenever genomes differing in this character are present.

Position of fertile pinnae on the leaf (terminal, mid-, lower), often a combination of parental features in fern hybrids (e.g., *Osmunda* hybrids, Wagner, 1962), was not a useful diagnostic character for *P. calirhiza* × *scouleri*. *Polypodium scouleri* and *P. calirhiza* fertile segments tend to be positioned terminally in the former, and may comprise all but the lower 1–3 segment pairs in the latter. Nonetheless, large variation occurs on parental leaves, particularly in *P. calirhiza*. Likewise, hybrid leaves show great variation ranging from few, often terminal segments with sori, to all segments producing sori.

However, the large variation in soral maturation on segments aptly indicates hybridization, with sori found in all developmental stages, albeit with malformed spores.

Fertile vein development and soral position frequently aid in delimiting fern hybrids. For example, *Polystichum lonchitis* produces fertile veins progressing from the midrib to the margin with sori midway and "dorsal" upon the vein whereas *P. acrostichoides* has fertile veins terminating at sori halfway between the margin and the midrib. A combination of parental traits occurs in fertile vein development of the hybrid *P. acrostichoides* × *lonchitis* with some sori terminal on fertile veins, other sori dorsal, and some sori lacking development (Wagner, 1962). In contrast, no distinction in soral position between *P. scouleri* and *P. calirhiza*, as well as the hybrid (when it occurs), is observed. Sori are terminal on fertile veins of parental species and *P. calirhiza* × *scouleri*. Close observation of parental species failed to determine differences in the manner by which fertile veins terminated, and, although *P. calirhiza* veins appear to end in a more reduced and less club-like form than those of *P. scouleri*, this difference may merely result from differences in leaf texture. Leathery hybrid leaves produce fertile veins more closely resembling *P. scouleri*, but, again, this may be an artifact of similar leaf textures. Sori do occur closest to the costa in *P. scouleri* whereas they are located midway between the costa and margin on *P. calirhiza* pinnae. The hybrid is highly variable producing sori both near the costa or midway between it and the pinna margin.

Leaf outlines of hybrids often blend those of parental individuals (Wagner, 1962), but this morphological feature is not transitional in *P. calirhiza* × *scouleri* leaves. Texture, leaf outline, pinna width and apex, as well as sinus angle and depth are all commonly useful for hybrid identification, but are not useful in delimiting *P. calirhiza* × *scouleri*. The resemblance of hybrid leaves to *P. scouleri* may be the most significant factor contributing to past difficulties in recognizing hybrid populations.

An unexpected discovery of the present study regards the average spore length for sampled *P. scouleri* plants from California. Published average spore length (Haufler *et al.*, 1993) for *P. scouleri* is less than 53 μm, whereas *P. calirhiza* spores exceed 58 μm. Indeed, *P. calirhiza* spores from Tank Hill and Mt. Sutro measured well within expected values whereas *P. scouleri* spores exceeded the expected average (Fig. 3). In an effort to explain the increased spore size, *P. scouleri* spores were harvested and measured from plants in Oregon (Hildebrand #3214, KANU), and, with an average of 55.3 μm, fell within the expected range.

Three explanations may account for the larger than average spores obtained from the California *P. scouleri* plants. Certainly, contamination of spore samples may have occurred. Although spores were removed directly from sporangia, *P. calirhiza* spores from dried material may have contaminated the *P. scouleri* plants measured. Two alternative possibilities are more difficult to assess. Environmental factors could account for the increase in average spore length found in California populations of *P. scouleri*. Spore

sizes of *Isoetes* species have been found to be strongly affected by environmental parameters including temperature, solar radiation, and elevation (Cox and Hickey, 1984). Finally, the increased average spore length observed in plants of *P. scouleri* from California could be explained by an increase in chromosome number. Although not always correlated to ploidy level, increases in spore size may indicate the formation of both auto- and allopolyploids. For example, average spore length was found to increase with ploidy level by a multiplier of 1.26 in species of neotropical *Polystichum* (Barrington *et al.*, 1986). The increase in average spore length for sampled California *P. scouleri* plants (ca. 15%) may correspond to an increased chromosome number via autopolyploidy.

To further explore this possibility, guard cells, often positively correlated with ploidy level (Barrington *et al.*, 1986), were compared between Oregon and California *P. scouleri* plants. Guard cell size was previously determined to predict allopolyploidy in the western *P. vulgare* complex. Barrington *et al.* (1986) found tetraploid *P. calirhiza* guard cells average 1.12 and 1.2 times larger than progenitors *P. californicum* and *P. glycyrrhiza*, respectively. Nevertheless, differences in guard cell length between California and Oregon *P. scouleri* populations were not observed, and decreased the possibility of an autopolyploid event in *P. scouleri* from sampled California populations.

Early cytological investigations by Manton (1951) of three plants identified as *P. scouleri* from Point Reyes, California revealed *n* pairs and *n* univalents at meiosis (vs. typically unpaired chromosomes in triploids) and suggested a closer genetic relationship between *Polypodium* tetraploids and diploids in western North America. It now seems most likely that the California plants Manton investigated were not *P. scouleri*, but *P. calirhiza* × *scouleri*. No cytological study has been completed for *P. scouleri* plants from Mt. Sutro and Tank Hill populations, and efforts to re-locate the Point Reyes hybrid populations have been unsuccessful.

The present study provides morphological and molecular evidence for the hybridization of *Polypodium scouleri* and *P. calirhiza* in California, and helps to secure the placement of the former in the western *P. vulgare* complex of North America. Questions remain regarding the California *P. scouleri* populations that merit further investigation. Future cytological studies of these populations, in conjunction with spore length measurements from fresh material, may aid in clarifying any remaining ploidy level conundrums.

Polypodium calirhiza* × *scouleri

Stem stout (5–15 mm diameter), occasionally whitish, acrid to slightly sweet-tasting. Rhizome scales uniformly brown to weakly bicolored with pale margins, lanceolate to lanceolate-ovate, symmetric, with occasional teeth on erose margins. Blades to 39 cm in length with a stout petiole to 3 mm in diameter. Lamina stiff and leathery, ovate-lanceolate, pinnatifid, usually widest at or just above the base, to 20 cm wide; sparsely scaly to

abaxially glabrescent; rachis sparsely puberulent adaxially. Rachis scales, concolorous, pale reddish brown, lanceolate-ovate; often adjacent, fused (from the base) to one third their length. Segments oblong to linear, usually more than 12 mm wide, with rounded apices, sparsely crenulate margins, midribs adaxially glabrous. Venation primarily free but with some anastomoses and irregularly formed aeroles. Sori oval to circular, but with widely varying development, usually closer to midrib than margins, 1–4 mm in diameter, producing malformed spores. Sporangiasters absent.

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Comparative Research of Gametophytes of *Olfersia alata* and *Olfersia cervina* (Dryopteridaceae)

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ABSTRACT.—The prothallial development of gametophytes of *Olfersia alata* and *Olfersia cervina* (Dryopteridaceae) is described and compared. Spores are monolete, ellipsoid, and with broadly winged perispore. Germination is *Vittaria*-type and the prothallial development is *Aspidium*-type. Adult gametophytes are cordiform-spatulate to cordiform-reniform, with marginal and superficial trichomes. Gametangia are of the type commonly found on leptosporangiate homosporous ferns. Differences between the two species of genus include size of the spores, width of the perispore, germination time, size of the trichomes, and time of formation of the gametangia. These two species share some features with some species of *Arachniodes*, *Cyrtomium*, *Dryopteris*, *Phanerophlebia*, and *Polystichum*, such as type of germination and prothallial development and trichomes. They differ from *Didymochlaena truncatula*, which has prothallial development of the *Adiantum*-type and lacks trichomes on the sexual phase.

The genus *Olfersia* Raddi (Dryopteridaceae), has two species: *Olfersia alata* C. Sánchez & García Caluff and *Olfersia cervina* (L.) Kunze. *Olfersia alata* is endemic to Cuba; its main characteristics are all sterile pinnae have decurrent bases, and fertile leaves which are smaller and have fewer pinna pairs than the vegetative leaves. It grows in mountainous mesophytic forests, between 350–400 m (Sánchez *et al.*, 1991). *Olfersia cervina* is widely distributed in the tropics, from Southern Mexico (Chiapas, Oaxaca, Veracruz), to Southeastern Brazil and the West Indies. In this species the bases of the sterile pinnae are not decurrent onto the rachis, and pinnae are short-petiolulate. It grows between 450–1000 m in damp tropical forests, on rocky and very shady banks (Moran, 1986, 1995; Riba and Pérez-García, 1999). Both taxa are usually terrestrial, rarely hemiepiphytic, with a short trailing rhizome. Leaves are markedly dimorphic, sori are exindusiate and linear to oblong, and spores are monolete, echinulate with a broad perispore.

This paper complements existing information about the morphogenesis of the gametophytic phase of dryopteriod ferns and, particulary, focuses on gametophytes of *Olfersia*. We hope to contribute in this way to the knowledge of the sexual phase of Mexican ferns.

MATERIALS AND METHODS

Spores of *O. cervina* were collected from living plants from the following site: Lote 69 in "Los Tuxtlas" Biological Station, between Laguna Azul and Laguna Seca, Mun. of San Andrés Tuxtla, in the state of Veracruz, Mexico; vouchers are in UAMIZ (*AMR 202* and *AMR 251*). Spores of *O. alata* were

collected by Carlos Sánchez and L. del Risco (*77825*) near Farallones de Moa, Farallón Redondo and La Escondida, Mun. Moa, Holguín province, Cuba; the voucher is in HAJM.

Fertile pinnae were kept in paper bags until spores were shed. Subsequently, the spores and shed material were passed through a sieve (with pores 0.074 mm in diameter) in order to eliminate traces of sporangia and indusia. Spores of each species were sown at an average density of 150–200 spores per cm^2 in two small pots with a mixture of black soil and organic matter and in 30 Petri dishes, 5 cm in diameter, containing Thompson's solution of mineral salts and agar on a sterile nutrient medium (Klekowski, 1969).

Petri dishes and pots were kept inside transparent plastic bags in order to avoid contamination and desiccation, with a photoperiod of 12 h light/darkness, with artificial light (75 Watt lamps, daylight) and a temperature of 23–25° C (Mendoza *et al.*, 1999a, 1999b). Two dishes were kept in darkness in order to determine photoblastism. After 100 days, none of the spores grown in darkness had germinated.

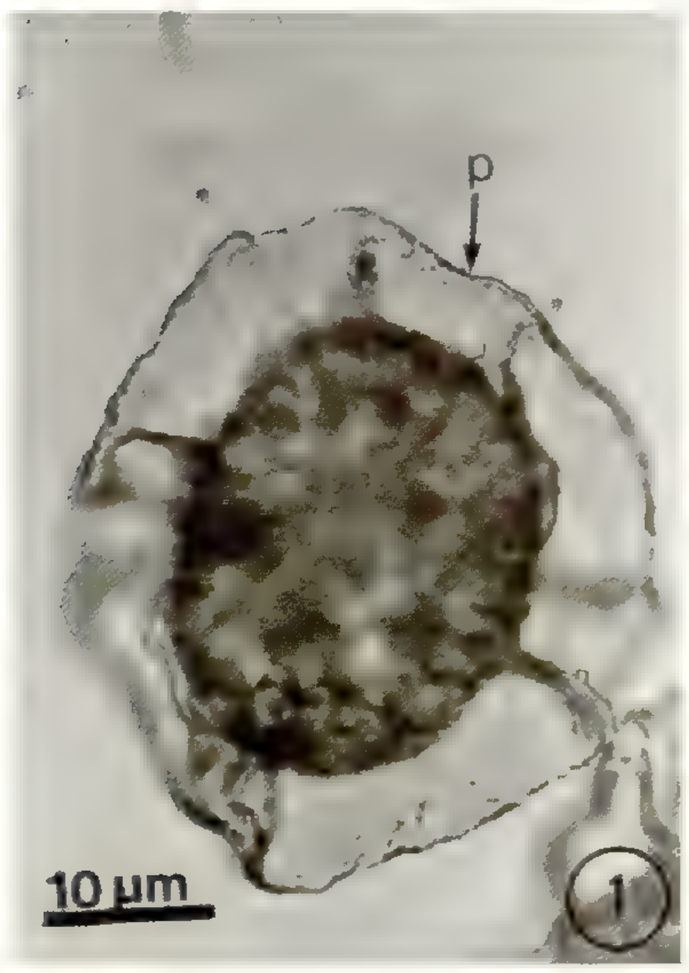
All pictures of microscopic material were taken from living material grown in the laboratory.

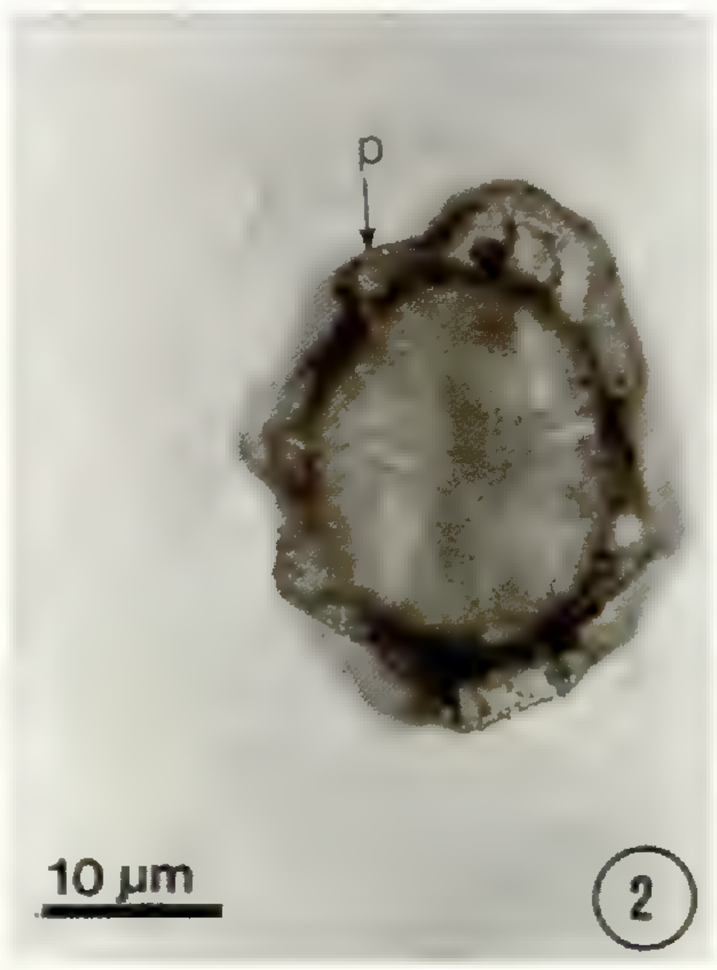
Results

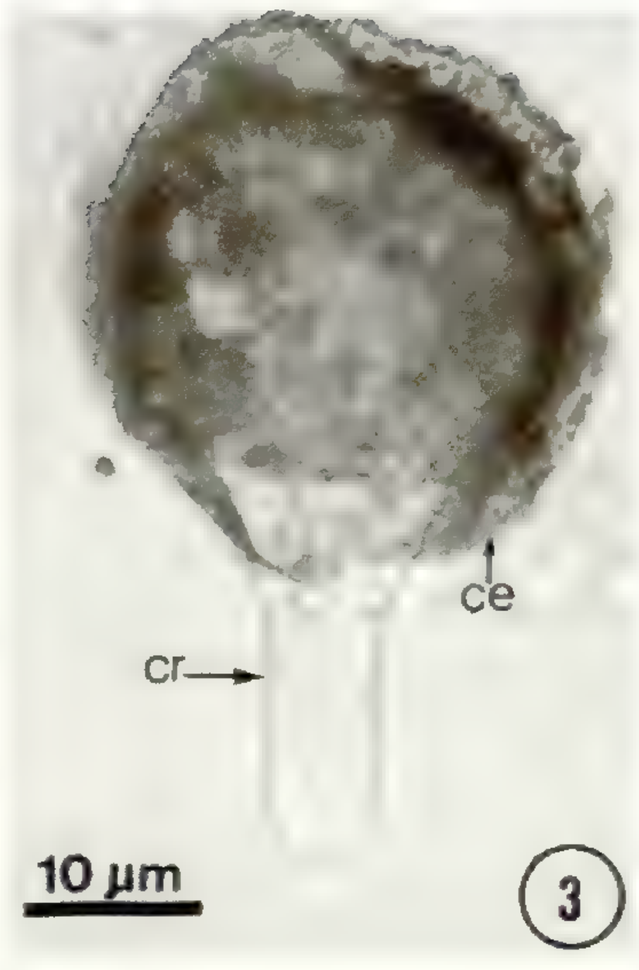
Spores of both species are monolete, nearly spherical, with a light brown perispore. Spores of *Olfersia alata* measure (64) 73 (83) × (49) 53 (55) μm, including the winged perine around the spore; the perispore measures (15) 16 (20) μm wide (Fig. 1). Spores of *O. cervina* measure (44) 48 (51) × (37) 39 (40) μm, also including the winged perine which measures (5) 6 (8) μm wide (Fig. 2). Spores of *O. alata* are larger than those of *O. cervina* primarily due to the size of the perispore. These measurements were obtained from an average sample of fifty spores per species.

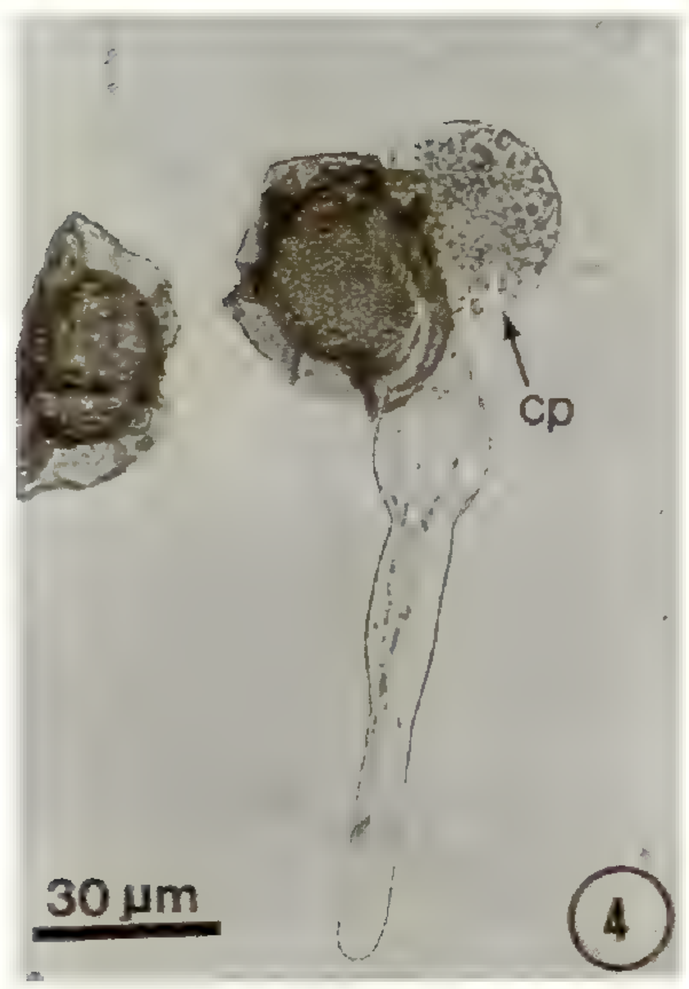
Germination is *Vittaria*-type (Nayar & Kaur, 1971) in both species. In *Olfersia alata* germination began 20–23 days after spores were sown, whereas in *O. cervina* it began 8–12 days after sowing. Gametophytes of both species first develop a rhizoid, which is short, hyaline, and without chloroplasts. The first prothallial cell is short and oval; division begins in this cell with a transverse wall and ultimately forms a short germ-filament, 2–4 cells long. This filament eventually ends in an apical trichome. During this stage of development, the spores retain their coat (Figs. 3–5).

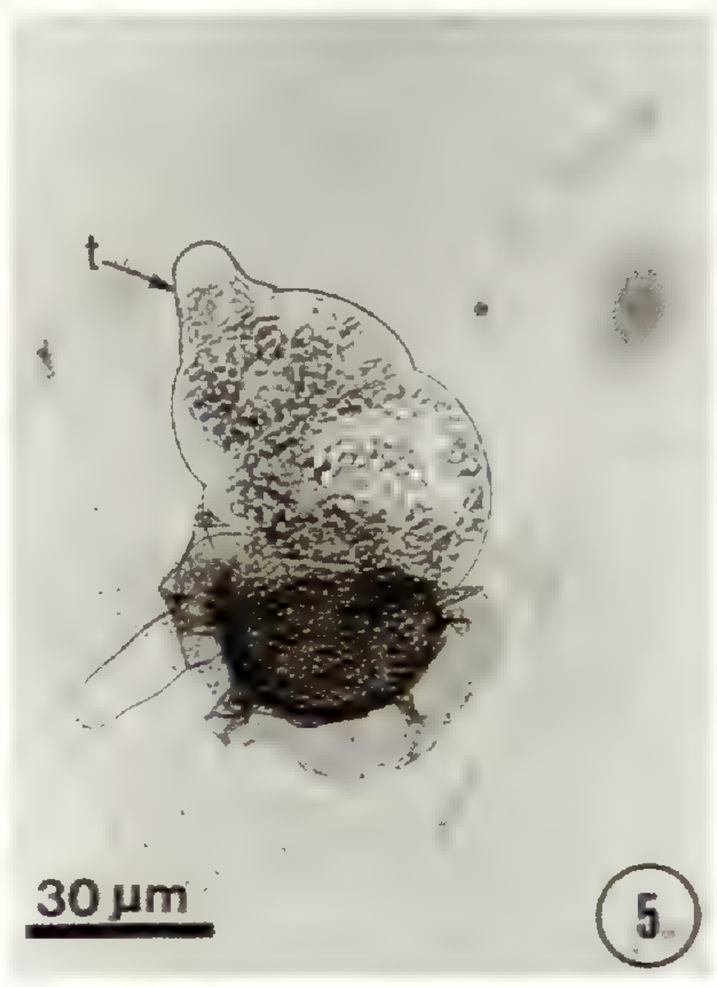
In *O. alata*, the prothallial plate begins to develop approximately 25 days after spore germination from intercalary cells of the filaments which undergo longitudinal divisions (Figs. 6–7). In some cases, the terminal cell of the filament, after producing a trichome, will divide longitudinally in such a manner that the trichome is placed over one of the daughter cells, which will remain inactive until other cells develops into a gametophytic plate. This plate, from which a meristematic cell will emerge, is usually asymmetric

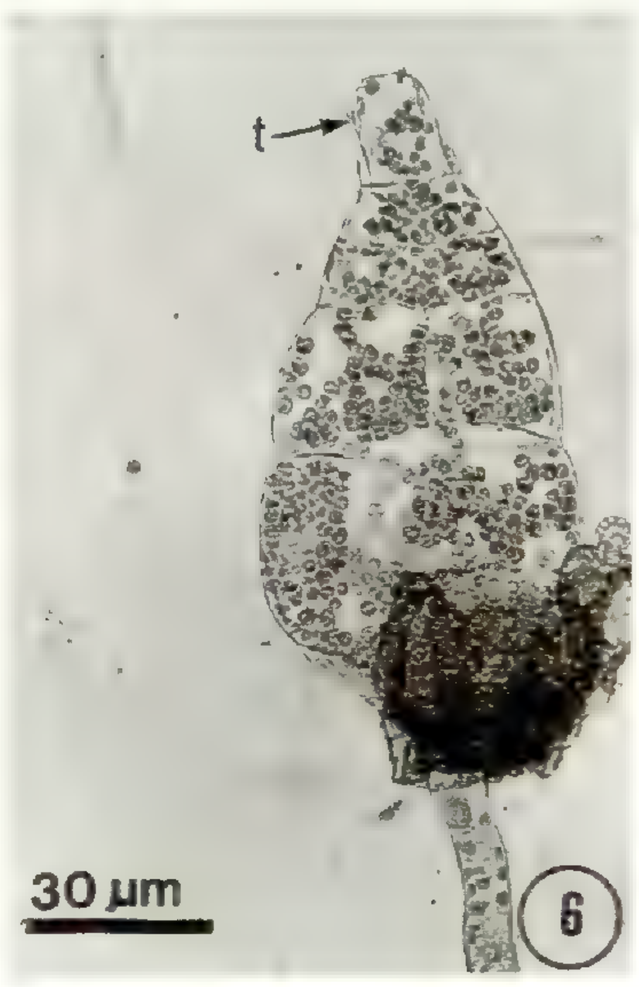


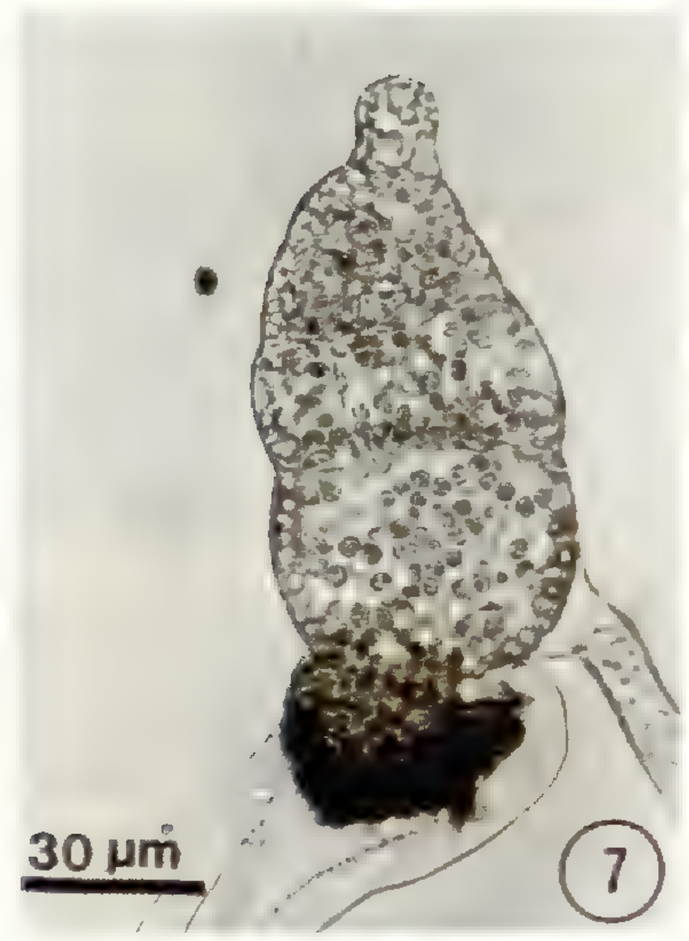


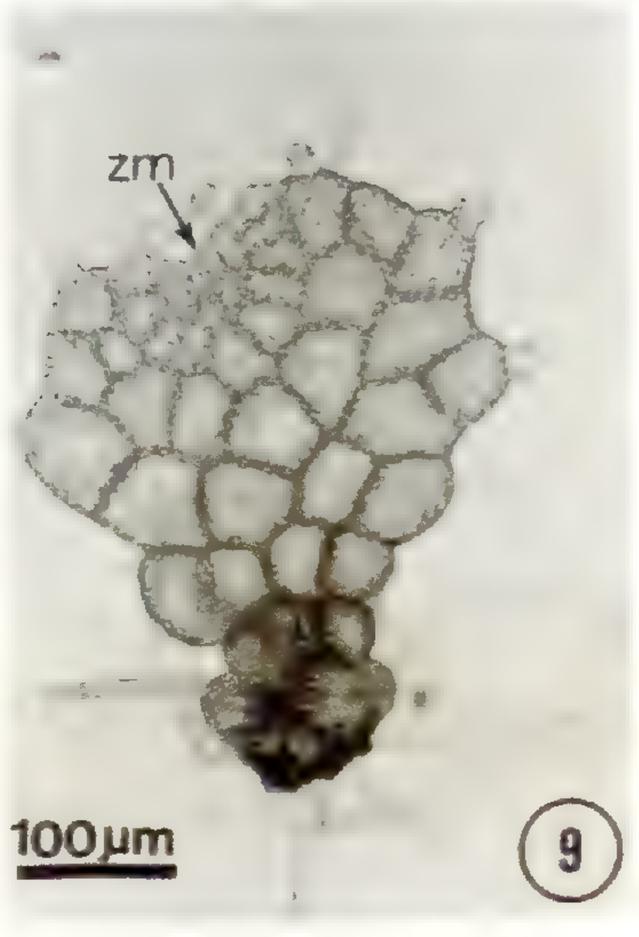




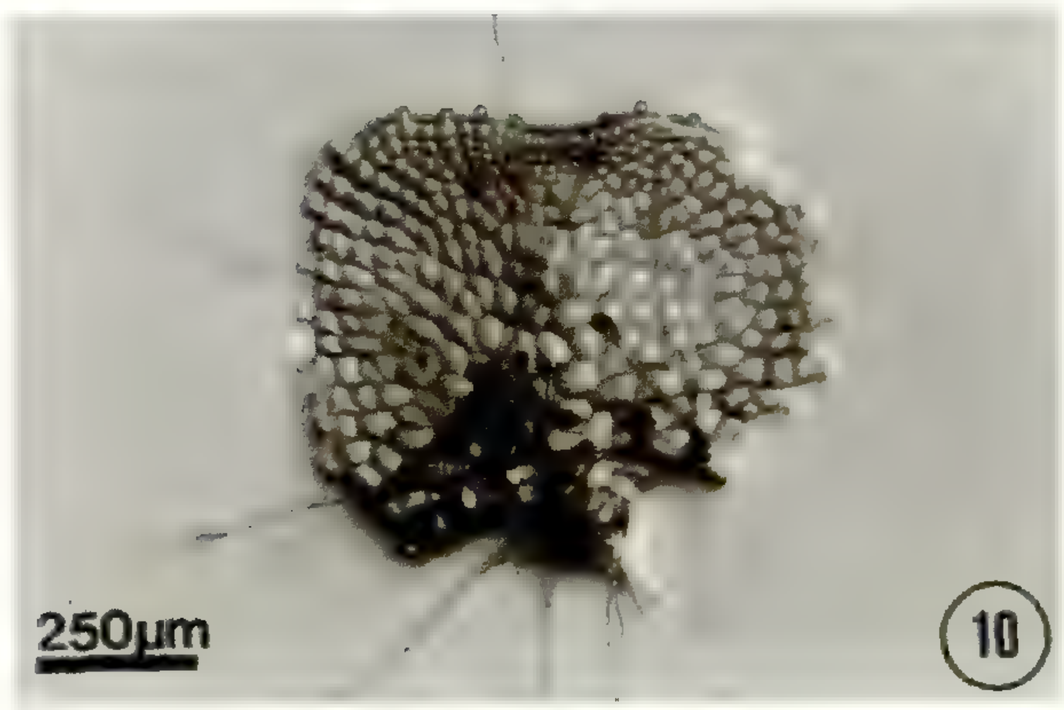


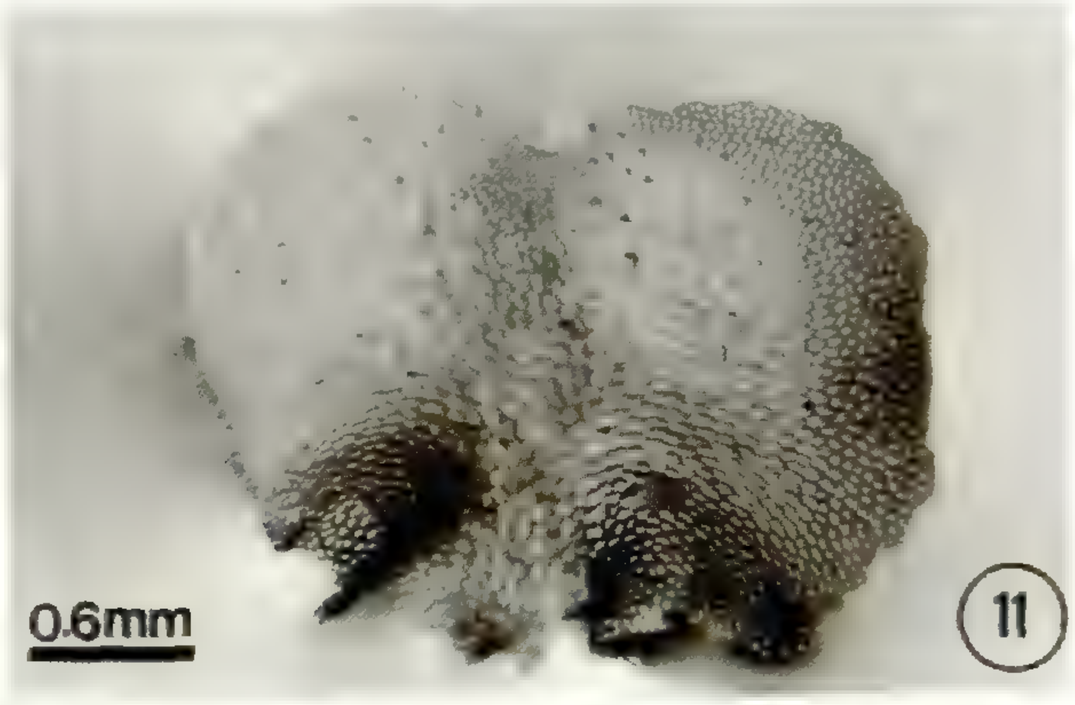


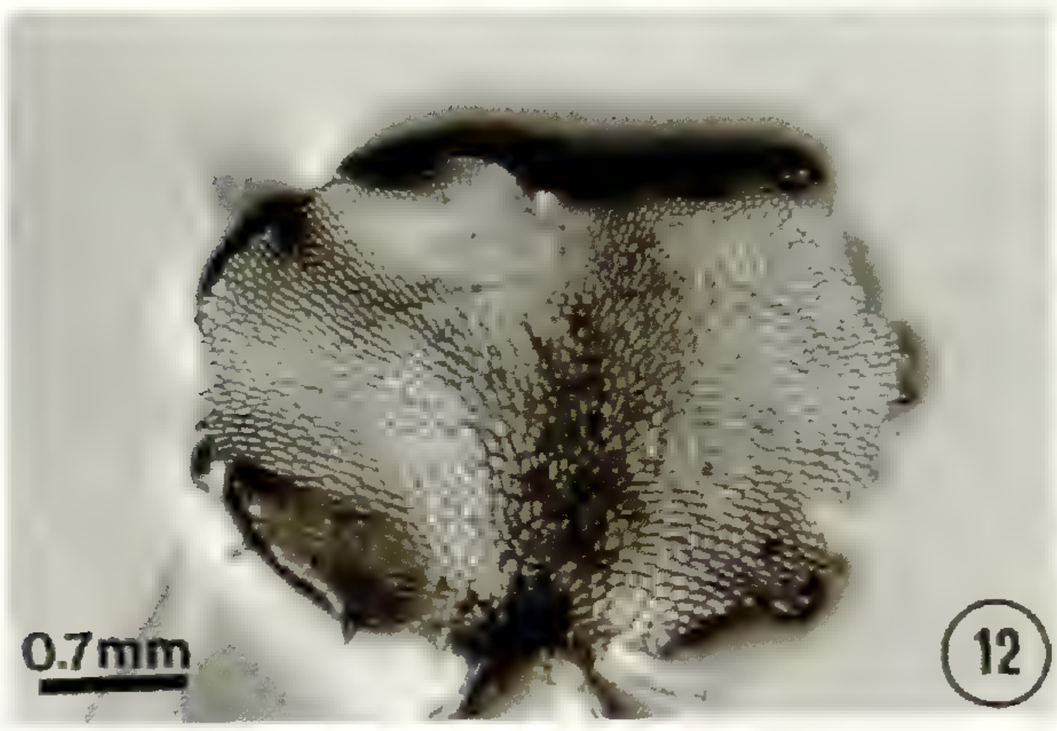


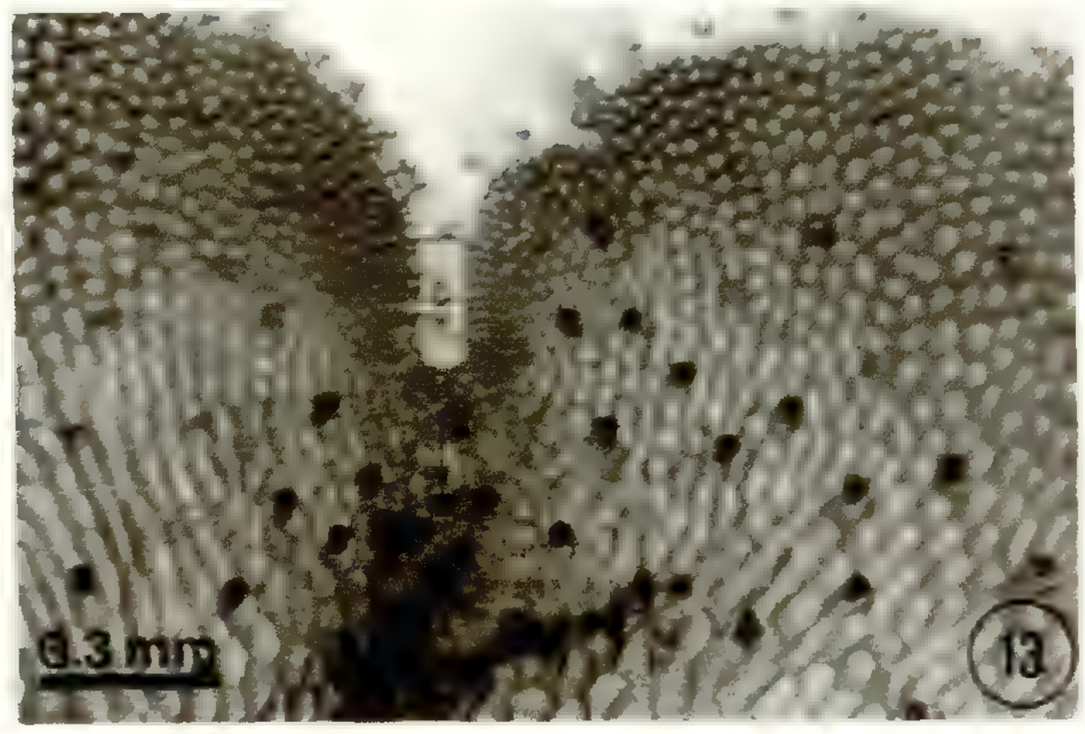


FIGS. 1–9. Spores, germination, and filamentous and laminar phases of *Olfersia*. 1. Spore, *O. alata*. 2. Spore, *O. cervina*. 3–4. Initial stages of germination. 3. *O. cervina* (8 days). 4. *O. alata* (22 days). 5–7. Germ filament. 5–6. *O. alata* (22 days). 7. *O. cervina* (22 days). 8–9. Young laminar phases. 8. *O. alata* (38 days). 9. *O. cervina* (35 days). ce = cover of spores, cp = prothallial cell, cr = rhizoid cell, p = perispore, t = trichome, zm = meristematic zone.



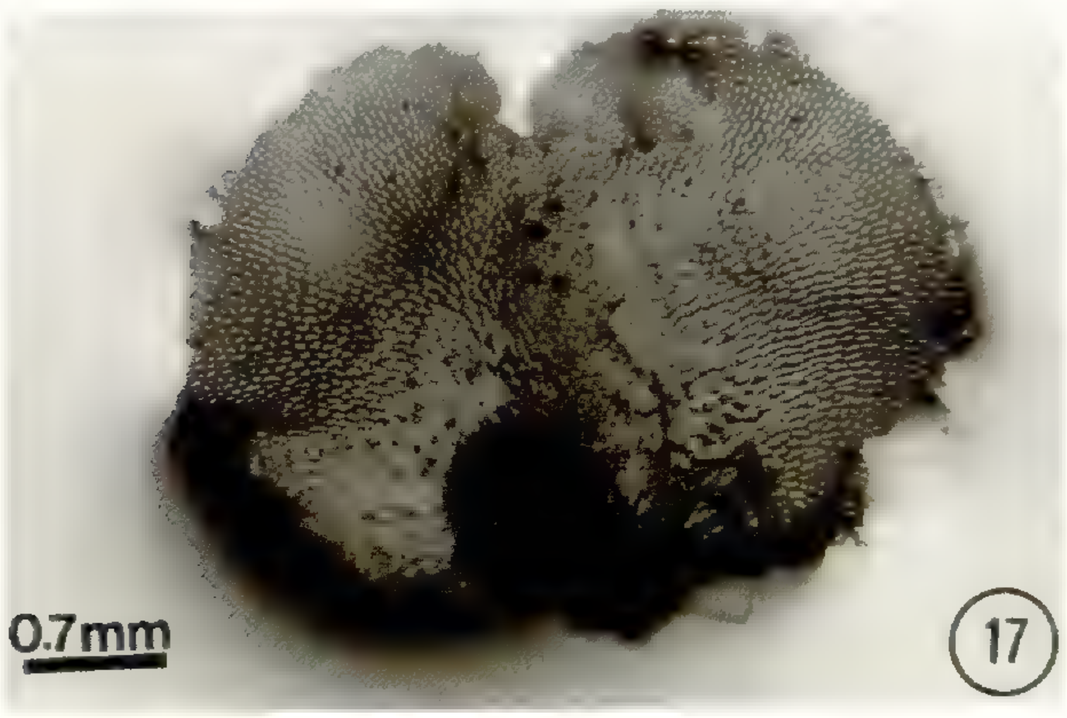


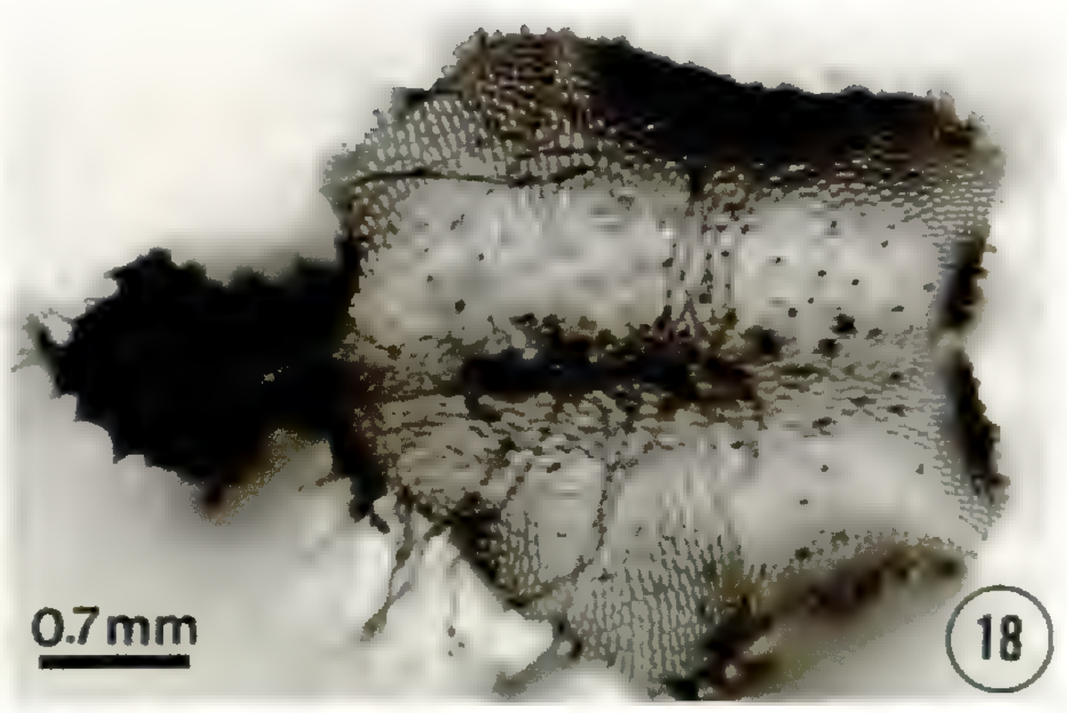


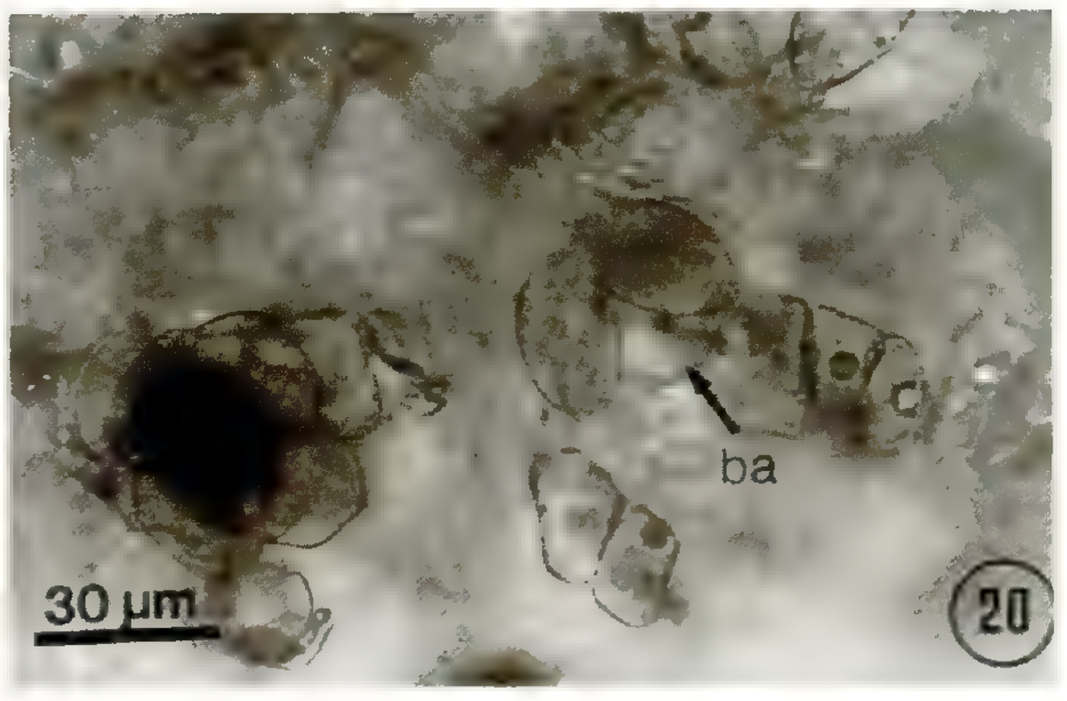


FIGS. 10–16. Laminar gametophytes and secretory, unicellular, capitate trichomes of *Olfersia*. 10–11. *O. cervina* (41 and 100 days). 12–13. *O. alata* (95 and 138 days). 14–16. Trichomes. 14. *O. cervina* (175 days). 15. *O. alata* (149 days). 16. *O. cervina* (175 days). a = archegonia, cse = extracellular secretion cover, t = trichome.

with an apex that continues to change and form a notch. Finally, after 90–100 days, the prothallial plate becomes cordate, the so-called Aspidium-type prothallial plate development (Nayar & Kaur, 1969; Figs. 8–10). Afterwards, a cushion bearing the gametangia forms and the adult gametophyte is cordiform-spatulate with many marginal and superficial trichomes. The formation







FIGS. 17–20. Adult gametophytes and gametangia of *Olfersia*. 17–18. Adult phases of *O. alata* (109–158 days). 19–20. Gametangia. 19. Antheridium, *O. cervina* (246 days). 20. Mouths of archegonium, *O. alata* (138 days). ba = mouth of archegonium, cb = basal cell, cm = ring cell, co = opercular cell.

of the prothallial plate in *O. cervina* takes less time, beginning on day 15. The pattern of prothallial development is the same as in *O. alata*. The first adult cordiform gametophytes are completely differentiated 60–80 days after the spores were sown (Figs. 12–13).

Trichomes are unicellular, capitate, and secretory (Fig. 14). In *O. alata* they measure approximately 36 µm long by 23 µm wide at the base. The apical third of the trichome is globose, 17 µm high by 24 µm wide, with a thin cover of extracellular secretion ca. 3 µm thick (Fig. 15). *Olfersia cervina* trichomes are 34 µm long by 20 µm wide at the base and the apical third of the trichome is globose, 21 µm high by 26 µm wide, with an extracellular secretion 8 µm thick (Fig. 16). These measurements are from mature trichomes, found at the middle basal region of the gametophytes.

The gametangia are typical of leptosporangiate homosporous ferns. They begin differentiating between days 120–244 in *O. cervina* while in *O. alata* they develop between days 100–150. Antheridia are distributed on the lower surface of the plate on the basal half of the cushion (Fig. 19). Antheridia are globose and consist of a basal cell, a ring cell, and an opercular cell. These three cells surround the androgenous cell.

In both species, the necks of the archegonia, have four tiers of neck cells. Archegonia are found on the central region of the plate, on the cushion, and

TABLE 1. Comparison of different stages of the prothallial development of *Olfersia alata*, and *O. cervina* with other genera and species of Dryopteridaceae.

	Spores	Type of germination	Filamentous phase	Type of prothallial development and adult form	Trichomes	Antheridium	Archegonium
[1,2]*Arachniodes*	Monolete, with perispore, measuring 30 42 μm	*Vittaria*	Long filaments (2-5 cells), with an apical trichome	*Aspidium*, cordiform gametophytes with lacerate margins	Unicellular, capitate, with a thin coat of extracellular secretion 2 :m	3 cells	4 rows of cells, each row with 4–6 cells
[1]*Cyrtomium*	Monolete with perispore, measuring 32 45 μm	*Vittaria*	Long filaments (2–5 cells), with an apical trichome	*Aspidium*, cordiform gametophytes, with lacerate margins	Unicellular, capitate	3 cells	4 rows of cells
[2]*Didymochlaena*	Monolete with perispore, measuring 30 37 μm	*Vittaria*	Short filaments (2–3 cells), apical trichome absent	*Adiantum*, cordiform-reniform gametophytes with entire margins	Absent throughout development	3–4 cells	4 rows of cells, each row with 4–6 cells
[3]*Dryopteris*	Monolete with perispore, measuring 36 51 μm	*Vittaria*	Long filaments (2–5 cells), with an apical trichome	*Aspidium*, cordiform-reniform gametophytes with lacerate margins	Unicellular, capitate	3 cells	4 rows of cells, each row with 4–5 cells
Olfersia alata	Monolete with a broadly winged perispore, measuring 53 73 μm	*Vittaria*	Short filaments (2–4 cells), with an apical trichome	*Aspidium*, spatulate-cordiform gametophytes with entire margins	Unicellular, capitate with an extra-cellular secretion coat 3 :m thick	3 cells	4 rows of cells, each row with 4–5 cells

TABLE 1. Continued.

	Spores	Type of germination	Filamentous phase	Type of prothallial development and adult form	Trichomes	Antheridium	Archegonium
O. cervina	Monolete with a winged perispore, measuring 39 48 μm	*Vittaria*	Short filaments (2–4 cells), with an apical trichome	*Aspidium*, spatulate-cordiform gametophytes with entire margins	Unicellular, capitate with an extra-cellular secretion coat 8 :m thick	3 cells	4 rows of cells, each row with 4–5 cells
[1]*Polystichum*	Monolete with perispore, measuring 34 45 μm	*Vittaria*	Long filaments (2–8 cells), without trichomes	*Aspidium*, cordiform gametophytes with lacerate margins	Unicellular, pappilate capitate, secretors and non secretors	3–4 cells	4 rows of cells
[4]*Phanerophlebia*	Monolete with perispore, measuring 25 33 μm	*Vittaria*	Long filaments (2–6 cells), with an apical trichome	*Aspidium*, spatulate-cordiform gametophyes with lacerate margins	Unicellular, capitate, with an extra-cellular secretion coat 1.5 :m thick	3–4 cells	4 rows of cells, each row with 4–6 cells

[1] Chandra & Nayar 1970, [2] Mendoza *et al.* 1999a, 1999b,[3] Pérez-García *et al.* 1999. [4] Mendoza, unpublished.

near the meristematic zone. The necks are oriented toward the basal region of the gametophytes (Figs. 13, 20). Two hundred days after sowing the spores, the young sporophytes had not yet formed.

Discussion and Conclusions

There is literature dealing with the morphology of the gametophytic phase of ferns closely related to *Olfersia*, of the Dryopteridaceae (both Old and New World), *e.g.*, *Arachniodes*, *Cyrtomium*, *Didymochlaena*, *Dryopteris*, and *Polystichum* (Atkinson, 1973; Chandra & Nayar, 1970; Cousens, 1975; Kaur, 1977; Mendoza et al., 1999a, 1999b; Pérez-García et al., 1999; Stokey & Atkinson, 1954).

Spores of *O. alata* average 73 × 53 μm, including the winged perispore; spores of *O. cervina* average of 48 × 39 μm. Spores of *O. alata* seem much larger, but in reality, if the perispore is not considered, the spores are 38 × 23 μm, and the winged perine is 16 μm wide or more in its widest part. Spores of *O. cervina* are 39 × 31 μm and the perispore is approximately 6 μm wide at its widest point and tends to be more spherical, which is an indication that the spores of *O. alata* are a little smaller than those of *O. cervina*. (Figs. 1, 2).

Both species share the same germination pattern, *Vittaria*-type, which is the most common type in ferns. In this type, the rhizoid develops first after the formation of a wall perpendicular to the polar axis of the spores. Eventually, the first prothallial cell divides by means of the formation of a perpendicular wall thus giving rise to two cells. The apical cell then divides again, giving rise to a short filament 2–4 cells long. The time for germination differs between these two species; spores of *O. cervina* germinate faster (8–12 days) compared to spores of *O. alata* (20–22 days).

Prothallial development in both species is of the *Aspidium*-type in which the germ filament commonly ends in a trichome, and the prothallial plate is formed by the activity of the intercalary cells of the filament. The adult gametophyte develops faster in *O. cervina* (60–80 days) than in *O. alata* (90–100 days).

Trichomes differ in size and in the thickness of the extracellular secretion; the longest ones, belongins to *O. alata* (36 × 23 μm), have a thinner extracellular secretion (3 μm), whereas trichomes of *O. cervina* are slightly shorter (34 × 20 μm) and have a thicker (8 μm) extracellular secretion.

Olfersia alata and *O. cervina* share features with the following dryopterid genera: *Arachniodes*, *Cyrtomium*, *Dryopteris*, *Phanerophlebia*, and *Polystichum* (Atkinson, 1973; Chandra and Nayar, 1970; Cousens, 1975; Mendoza *et al.*, 1999b; Pérez-García *et al.*, 1999). These genera all have monolete spores with perispore, a *Vittaria*-type germination pattern and an *Aspidium*-type prothallial development. However the two *Olfersia* species differ from the rest in the shape of their trichomes, which are short and wider at the base, capitate, with a globose apex, and with a dense extracellular secretion.

The gametophyte margins are entire in *Olfersia*, compared with the lacerate margins of species of the other genera. These other genera also have longer, capitate trichomes, with very thin extracellular secretions distributed on the lacerate margins and on the surfaces of the plate (Table 1).

Olfersia alata and *O. cervina*, together with the above mentioned taxa, share some features with *Didymochlaena truncatula*, such as the monolete spores and *Vittaria*-type germination. This last species differs from the rest in that is has a prothallial development of the *Adiantum*-type, characterized by a differentiation of an apical meristematic cell during the early stages of the plate's formation. The gametophytes of *Didymochlaena*, are completely glabrous throughout their development, in contrast to the other species of Dryopteridaceae mentioned.

Based on our results, we conclude that *Olfersia alata* and *O. cervina* share characteristics such as the *Vittaria*-type germination pattern, *Aspidium*-type prothallial development, and unicellular capitate trichomes with a uniform extracellular secretion. These same characteristics are characteristic of species of *Arachniodes*, *Cyrtomium*, *Dryopteris*, *Phanerophlebia*, and *Polystichum* (Table 1). The most common feature of all of these genera is the development of an apical trichome during the filamentous stages of prothallial development. Gametophytes of *Didymochlaena truncatula* differ from these genera in having prothallial development of the *Adiantum*-type and lacking trichomes. Finally, with the exception of *Didymochlaena truncatula* we did not find important differences among the different taxa of the Dryopteridaceae.

Acknowledgments

This paper is part of an MSc (Plant Biology) thesis. Morfogénesis de la fase sexual de pteridofitas mexicanas, familia Dryopteridaceae, ("Morphogenesis of the sexual phase of Mexican Pteridophytes, family Dryopteridaceae"), written by the first author, developed under the supervision of the co-authors of this paper. We especially thank Carlos Sánchez Villaverde for sending spores of *Olfersia alata*, without which this research would not have been possible; we thank Alan Smith for extremely valuable comments to the manuscript, we thank Jorge Lodigiani for his photographic assistance, and the anonymous reviewers for their suggestions and criticisms.

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Shorter Notes

***Botrychium hesperium* in the Wallowa Mountains of Oregon.**—The Wallowa Mountains of northeastern Oregon boast the greatest fern diversity in the state. We reported 47 taxa in the range (Zika & Alverson, Amer. Fern J. 86: 61–64. 1996), which included 14 taxa of *Botrychium*. A number of elements from the Rocky Mountains are found in Wallowa County, to which we can now include *Botrychium hesperium* (Maxon & R. T. Clausen) W.H. Wagner & Lellinger, an addition to the Oregon flora (Wagner & Wagner, Ophioglossaceae in *Flora of North America, Vol. 2*, Oxford Univ. Press, 1993).

Botrychium hesperium is restricted in the Wallowa Mountains to a narrow elevational band in the Lostine River drainage, between 1535–1660 meters, where steep canyon walls shade the valley floor from direct sunlight early and late in the day. It is found in mesic meadows or forest edges, in full sun or partial shade, at all aspects, but only on gentle slopes or flats on the valley floor. It has yet to be located on steep slopes at higher elevations. The forests are primarily *Pinus contorta* Dougl. *ex* Loud., with low wet areas dominated by *Picea engelmannii* Parry *ex* Engelm. Associated herbs include: *Achillea millefolium* L., *Agoseris aurantiaca* (Hook.) Greene, *Antennaria rosea* Greene, *Calamagrostis rubescens* Buckl., *Carex concinnoides* Mack., *C. geyeri* Boott, *C. hoodii* Boott, *Elymus glaucus* Buckl., *Erigeron compositus* Pursh, *Festuca occidentalis* Hook., *Fragaria virginiana* Duchesne, *Gentiana amarella* L., *Hieracium albiflorum* Hook., *Linnaea borealis* L., *Sedum stenopetalum* Pursh subsp. *stenopetalum*, *Senecio pseudaureus* Rydb., and *Viola adunca* Sm. It grows with *Botrychium lanceolatum* (Gmel.) Ångstr. subsp. *lanceolatum*., *B. minganense* Victorin, *B. paradoxum* W. H. Wagner, *B. pedunculosum* W. H. Wagner, and *B. pinnatum* St. John. The sites are valley bottom Quaternary surficial deposits, locally reworked by the Lostine River or small tributaries. Adjacent slopes are sedimentary bedrock in the Triassic/Jurassic Hurwal Formation. In places the upper west wall of Lostine Canyon is granite, and the east wall is pure limestone of the Martin Bridge Formation. It is possible that all or most of the *Botrychium* populations are influenced by basic or circumneutral groundwater percolating through calcareous glacial till or morainal debris. It may be no coincidence that the richest diversity and greatest abundance of *Botrychium* species are found in the calcareous canyons of the Wallowa Mountains, rather than in the granitic or volcanic basins.

We are aware of four extant populations of *Botrychium hesperium* in the Wallowas. The Oregon range of the species is included in ca. 5.5 km of river valley. The total known number of plants at this time is less than 100, and they face threats from fire suppression, pack animal grazing, wood-cutting, and recreation-associated activities, despite the fact that most or all plants are within the Lostine River Wild and Scenic River corridor, a part of the Eagle Cap Ranger District of the Wallowa-Whitman National Forest.

Collections of *Botrychium hesperium* were first made in 1981 (*W. H. Wagner 81130* MICH), with later collections in 1991 (*Zika & Alverson 11295* WTU), 1992 (*Zika & Alverson 11794* WTU), 1993 (*Wagner et al. 93047* MICH) and 1996 (*Zika & Alverson 12908* OSC). We were puzzled by these plants for many years, and thought they might represent an undescribed taxon, related to *B. hesperium*, but with slightly angular upper pinnae and shorter basal pinnae. This was a false impression, based in part on the large Wallowas plants growing in sheltered or partly shaded sites, and based on a limited sample of *B. hesperium* from Oregon and elsewhere. To get a better idea of variation in *B. hesperium*, we studied large living populations in Montana, Arizona and Colorado. Finally, as we saw more Oregon plants, we concluded they were part of the natural variation of *B. hesperium*, united by their grayish-green color, exaggerated and asymmetrical basal pinnae, broad rounded upper pinnae, and ample sporophores.

We are pleased to acknowledge our funding sources for fieldwork: the Native Plant Society of Oregon, the Oregon Natural Heritage Program, and the Wallowa-Whitman National Forest. We are grateful for specimens and discussions of B. hesperium, provided by Peter Root, Peter Lesica, Kathy Ahlenslager, and Don Farrar.—PETER F. ZIKA and EDWARD R. ALVERSON, Herbarium, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, and WARREN H. WAGNER (deceased) and FLORENCE S. WAGNER, Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109.

A Binomial for the Hybrid *Polypodium* of Eastern North America.—Two species of *Polypodium* (Polypodiaceae) occur in eastern North America, the diploid *P. appalachianum* Haufler & Windham and the tetraploid *P. virginianum* L. These species hybridize, producing a sterile triploid recognized by its abortive spores and intermediate morphology. The differences between these three taxa are well described by Haufler and Wang (Amer. J. Bot. 78:624–629. 1991) and Haufler and Windham (Amer. Fern J. 81:7–23. 1991). The triploid hybrid so far has been found only on the Appalachian Plateau where *P. appalachianum* and *P. virginianum* are sympatric. The hybrid has been documented so far in Ontario, Canada and eight states: New Hampshire, New Jersey, North Carolina, Ohio, Pennsylvania, Tennessee, Vermont, and Virginia (Evans, Research Div. Monograph 2. Virginia Polytechnic Inst. and State Univ., Blacksburg, VA. pp. 117–146. 1970; Haufler & Wang, *op. cit.*; Montgomery, Bartonia 59:113–117. 1996). Kentucky and West Virginia can be added to this distribution, based upon specimens at OS and WVU, respectively. The hybrid likely will be documented in other states and provinces as well. Indeed, the triploid may prove rather frequent, as shown for New Jersey and Pennsylvania by the work of Montgomery cited above.

It seems appropriate and practical that this widespread hybrid have a binomial. Perhaps providing this taxon with an epithet may raise botanists' awareness of this taxon and spur future discoveries and understanding of this hybrid.

Polypodium ×incognitum Cusick, *hybr. nov.*—Holotype: Ohio, Meigs County, sandstone exposures on mesic slope above Leading Creek, Co Rt 10, 0.25 mi (0.02 km) SW of Twp Rt 27, N of Dexter, Sect 6, Salem Twp, 6 Aug 1985, *Cusick 24620,* OS; Isotypes, MICH, MU, NY.

Hybrida e Polypodium appalachianum et P. virginianum exorta, aliis characteribus inter parentes media, sporis abortivus.

My research was supported in part by the Division of Natural Areas and Preserves, Ohio Department of Natural Resources.—Allison W. Cusick, Division of Natural Areas and Preserves, Ohio Department of Natural Resources, 1889 Fountain Sq. Ct., F-1, Columbus OH 43224.

***Lycopodium lagopus* New in West Virginia.**—West Virginia is a southern outpost for many boreal species (e.g. *Larix laricina* in Preston County) that were stranded in the state's highlands and arctic-like bogs following the last glacial retreat (P.D. Strausbaugh and E.L. Core, *Flora of West Virginia*, Morgantown WV, Seneca Books, 1997). Along the Allegheny Front, elevations reach 1482 m (Spruce Knob) and there are ten peaks over 1430 m. *Lycopodium lagopus* (Laestradius *ex* C. Hartman) G. Zinserling *ex* Kuzeneva-Prochorova, (Fl. Murmansk Obl. 1:80, 1953), generally more northern in its distribution, was recently located here as well. A small, but thriving population grows on the site of a coal strip mine, now used as a cross country ski trail in Blackwater Falls State Park, Tucker County, at an elevation of about 1070 m. Its sister species, *L. clavatum*, is also here in abundance, but the two lycopods remain distinct; *L. lagopus* features single strobili on slender peduncles, a more compact growth habit, more appressed and shorter leaves, and sporophylls that taper gradually to a hair tip.

Lycopodium lagopus (formerly *L. clavatum* var. *monostachyon* Hooker and Greville) goes by the apt common name "one-cone club-moss" (*Flora of North America*, New York, Oxford Univ. Press, 1993). It shares many characters with the common club moss, *L. clavatum*, *e.g.*, general growth and branching patterns, stalked strobili, and hair-tipped leaves, but *L. clavatum* has multiple strobili (typically two) on most of its peduncles, spreading and longer leaves, and sporophylls that end abruptly in hair tips. No hybrids are documented between these closely related species, nor, for that matter, between any species in the genus *Lycopodium s.s.* This is in sharp contrast to the many hybrids described since 1956 within the related genera *Lycopodiella*, *Huperzia*, and *Diphasiastrum* (J. Eiger. Biol. Rev. City Coll. 18:17–22, 1956; *Flora of North America*).

As a boreal plant, *L. lagopus* occurs from Alaska to Newfoundland, Green-

land, Scandinavia, and northern Eurasia. In the contiguous 48 states it has been reported from Maine, Michigan, Minnesota, Wisconsin, New York, New Hampshire, Vermont, and now West Virginia. Michigan would be the closest known neighbor of the West Virginia population, about 650 km distant (*Flora of North America*, cited above). The West Virginia site is the flat top of an abandoned coal strip mine within Blackwater Falls State Park, near the town of Davis. The park was established in 1937, and mining operations within its borders ceased prior to that. Since that time a remarkable assemblage of plants has reclaimed the coal spoils piled along the mine highwall. In cool ravines there is *Tsuga canadensis*, while on the open, sunny, coal-strewn areas *Picea rubens*, *Acer rubrum*, *Rhododendron maximum*, *Kalmia latifolia*, and *Vaccinium* species dominate the woody flora. Three orchids are prominent among the herbaceous plants- *Cypripedium acaule*, *Platanthera clavellata*, and *Spiranthes cernua*. Many grass, sedge, and *Sphagnum* species grow in the boggy soils near two ponds at the intersection of the Dobbin House and Woodcock ski trails in the area of *L. lagopus*. An impressive group of fern allies has also reclaimed this disturbed, acidic habitat, including *Lycopodiella inundata* on moist soil near the ponds. *Diphasiastrum digitatum*, *D. tristachyum*, and their hybrid *D.* ×*habereri* are abundant on exposed tailings. *Lycopodium obscurum*, *L. dendroideum*, and *L. hickeyi* also occur along the wooded edges. And, the aforementioned *L. clavatum* is found in nearly all surrounding habitats. Several *Dryopteris* species and *Pteridium aquilinum* (with some rare, fertile colonies) are also common in the immediate area. *Asplenium montanum* grows on granite rocks along the Pase trail nearby, and *Vittaria appalachiana* (Appalachian gametophyte) is spreading under a sandstone ledge near the prominent waterfall for which the 683 hectare (1688 acre) park is named.

The *Lycopodium lagopus* colony consists of about a dozen long rhizomes, three occurring on exposed soil adjacent to the Woodcock Trail and the rest in a protected area about 6 m into low spruce woods beyond the trail. The colony is probably clonal and is quite fertile, nearly all upright shoots bearing characteristic single strobili when the site was surveyed in mid-July, 2001. A voucher specimen of one fertile, upright shoot was collected for deposit with the herbarium of the Carnegie Museum in Pittsburgh, PA (sheet No. 494379, CM). The origin of the "one-cone club-moss" here is uncertain, but it is hardly the only disjunct, rare pteridophyte known in West Virginia. The western species *Asplenium septentrionale* and *Cheilanthes eatonii* (*C. castanea*) occur on shale in Hardy and Monroe Counties (W. H. Wagner, Jr., Ann. Missouri Bot. Gard. 59:203–217, 1972).—JOAN EIGER GOTTLIEB, 2310 Marbury Road, Pittsburgh, PA 15221.

***Marsilea mutica* in Virginia.**—Of the six species of water-clover, *Marsilea*, described in Flora of North America, five are native and *M. quadrifolia* L. is introduced in much of the northeastern United States. Johnson (pp 332–333, in FNA ed. comm., *Flora of North America, vol. 2*, 1993) suggested that *M. quadrifolia* has been deliberately planted as a curiosity because many localities are artificial bodies of water. A seventh species, the paleotropical *Marsilea minuta* L., has been collected in Florida (Burkhalter, Sida 16:544–549, 1995). We document here the apparent establishment of a previously unreported water-clover in southeastern Virginia, *M. mutica* Mettenius, which, unlike most species in the genus, can be readily identified in sterile condition by its two-toned leaves (Fig. 1). This station represents the first documented occurrence of this Australasian species in North America.

A small but vigorous population was discovered on 20 October 2001 growing in shallow water (<1.0 m depth) and adjacent muddy shores for 10 m along Cooper's Ditch, a canal dug through non-tidal forested wetlands in the early 1980's as a stormwater in the city of Chesapeake. Plants were restricted to shallow water and associated with *Utricularia* sp., *Hydrilla verticillata* (L.f.) Royle, *Hydrocotyle verticillata* Thunb., *Myriophyllum pinnatum* (Walter) BSP, and filamentous algae. Like other species in the genus, floating leaves were much larger than leaves on terrestrial plants (Fig. 1). Rhizomes were rooted. No sporocarps were present. Collection data follows: Virginia: City of Chesapeake, NE side of Hillwell bridge across Cooper's Ditch, Coordinates: 36°42′01″N, 76°13′32″W, 20 October 2001, *L. J. Musselman and D. A. Knepper, 2001-36* (ODU).

Winter hardiness in this species is not known. Nothing is known about the invasiveness of this species and the population should be monitored to measure its persistence and its spread.—David A. Knepper, U S Army Corps of Engineers, Fort Norfolk, Norfolk, VA 23510-1096; David M. Johnson, Department of Botany-Microbiology, Ohio Wesleyan University, Delaware,

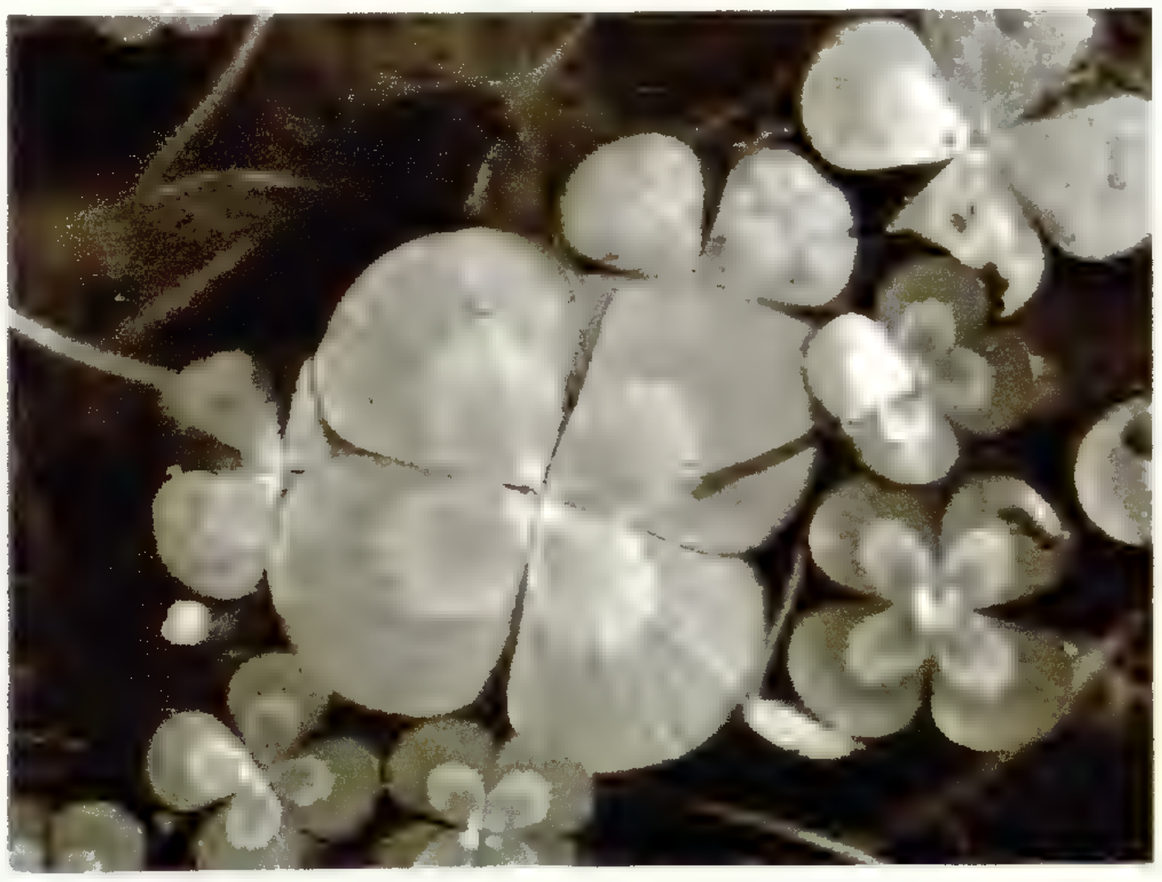

Fig. 1. Habit view of *Marsilea mutica*.

OH 43015; and LYTTON JOHN MUSSELMAN, Department of Biological Sciences, Old Dominion University, Norfolk, VA 23529-0266.

3,8-Di-*C*-arabinosylluteolin, a new flavonoid from *Pteris vittata*.—In spite of the fact that fern flavonoids are of chemotaxonomic interest, little is known of the distribution of these compounds in some fern families (e.g. Pteridaceae). Previous work on the flavonoids of *Pteris vittata* L. (Pteridaceae) has led to the identification of an anthocyanin (luteolinidin 5-*O*-glucoside) by Harborne (Phytochemistry 5:589–600, 1966). In addition, acid hydrolysis of extracts of this fern has led to the identification of kaempferol, quercetin, leucocyanidin and leucodelphinidin by Voirin (Ph. D. thesis, University of Lyon, p. 151, 1970). More recently 3-*C*-(6‴-acetyl-cellobiosyl)-apigenin (Amer. Fern J. 89:217–220, 1999) and 6-*C*-cellobiosylisoscutellarein 8-methyl ether together with quercetin 3-*O*-glucuronide and rutin (Amer. Fern J. 90:42–47, 2000) have been identified by Imperato and Telesca. Three kaemperol glycosides (3-*O*-glucoside, 3-*O*-glucuronide and 3-*O*-(X″,X″-di-protocatechuoyl)-glucuronide) together with quercetin 3-*O*-(X″,X″-di-protocatechuoyl)-glucuronide have been found in this fern by Imperato (Amer. Fern J. 90: 141–144, 2000).

In the present paper a new *C*-glycosylflavone (identified as 3,8-di-*C*-arabinosyl-luteolin (I)) and 6-*C*-arabinosyl-8-*C*-glucosylluteolin (II) have been isolated from *Pteris vittata* L. growing in the Botanic Garden of the University of Naples. This fern has been identified by Dr. R. Nazzaro (University of Naples); a voucher specimen (149.001.001.01) has been deposited in the Herbarium Neapolitanum (NAP) of the University of Naples.

Flavonoids (I and II) were isolated from an ethanolic extract of aerial parts of *Pteris vittata* L. by preparative paper chromatography in BAW (*n*-butanol-acetic acid-water, 4:1:5, upper phase), 15% HOAc (acetic acid) and BEW (*n*-butanol-ethanol water, 4:1:2.2). Further purification was carried out by Sephadex LH-20 column chromatography eluting with methanol.

Color reactions (brown to yellow in UV+NH_3), ultraviolet spectral analysis in the presence of usual shift reagents (λ_{max} (nm) (MeOH) 258, 272, 348; +NaOAc 283, 322 (sh), 402; +NaOMe 266, 281, 340 (sh), 407 (increase in intensity); +$AlCl_3$ 277, 299 (sh), 331 (sh), 426; +$AlCl_3$/HCl 280, 300 (sh), 358, 388) and chromatographic behaviour (R_f values on Whatman No 1 paper: 0.15 in BAW; 0.31 in 15% HOAc; 0.12 in H_2O) suggested that flavonoid (I) may be a flavonoid glycoside with free hydroxyl groups at positions 5, 7, 3′ and 4′. Since treatment with 2N HCl (2 hr at 100°C) failed to produce an aglycone, flavonoid (I) may be a *C*-glycosylflavonoid. Electrospray mass spectrum (ESMS) showed a pseudomolecular ion at m/z 573 $[(M+H)+Na]^+$, an ion at m/z 595 $[(M+H)+2\ Na]^+$ and an ion at m/z 1123 $[(M \times 2)+ Na+H]^+$ which corresponds to a dimer. These data suggest that flavonoid (I) is a di-*C*-pentosylluteolin. ^1H NMR spectrum (DMSO-d_6) showed signals at δ 3.11–3.91 (ten sugar protons, m), δ 4.61 (1H, d, J=8 Hz, anomeric proton), δ 4.68 (1H, d, J=8 Hz, anomeric proton), δ 6.27 (1H, s, H-6), δ 6.93 (1H, d, J=8.8

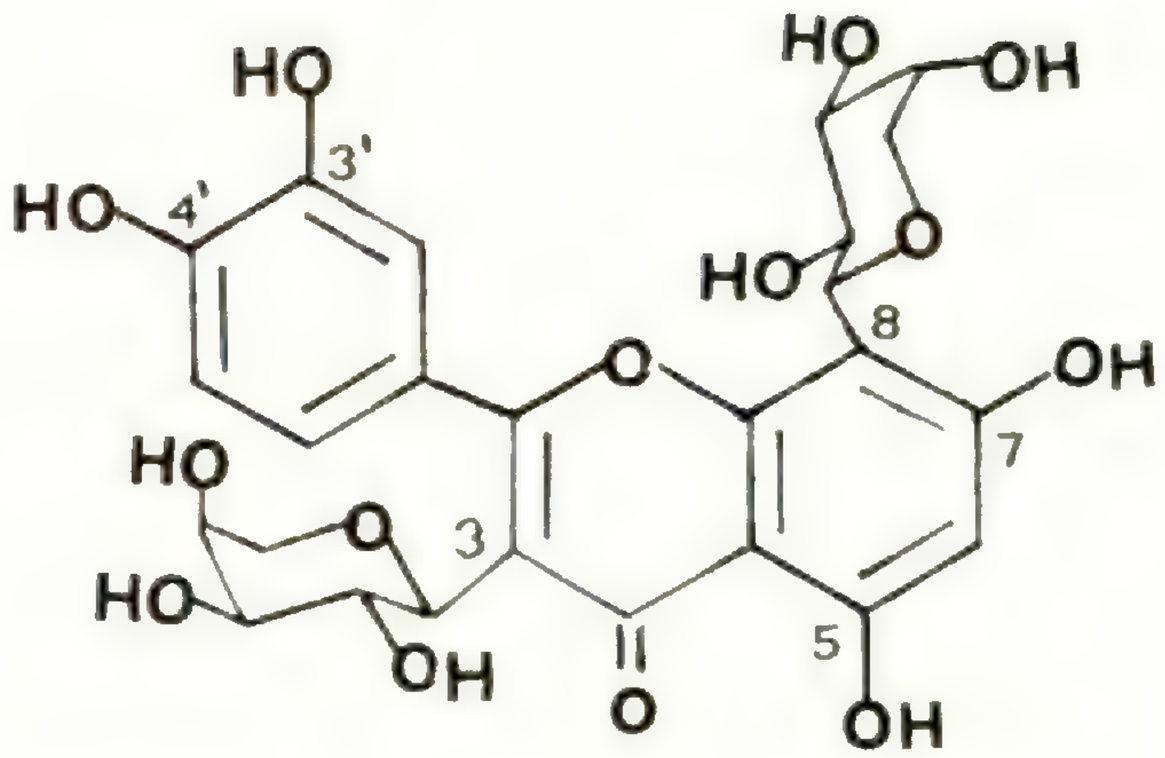

FIG. 1.

Hz, H-5′), δ 7.39 (1H, dd, J=2.0 and 8.8 Hz, H-6′) and δ 7.40 (1H, d, J=2 Hz, H-2′). These data suggest that flavonoid (I) is a luteolin 3,8-di-*C*-pentoside.

Wessely-Moser isomerization (3N HCl; 3 hr at 100°C) gave a mixture in which flavonoid (I) and four isomers were detected by paper chromatography in BAW; these isomers were not present in sufficient amount to allow characterization. ^{1}H NMR spectrum (DMSO-d_6) of the above mixture showed a singlet at δ 6.56 (H-8) and a singlet at δ 6.26 (H-6) confirming that flavonoid (I) is a 3,8-di-*C*-glycosylflavone. Since flavonoid (I) gave at least four isomers, arabinose may be attached at C-3 and/or C-8 because *C*-arabinosylflavones on acid treatment undergo pyranose-furanose isomerization and α-linkage-β-linkage isomerizatiion of *C*-glycosidic link as described in a review of Chopin et al. (pp. 449–503 *in* J.B. Harborne and T. J. Mabry eds., *The Flavonoids: Advances in Research*, Chapman and Hall, London, New York, 1982). Treatment of flavonoid (I) with 2,2-dimethoxypropane and 6N HCl-dioxan in dry dimethylformamide according to Jarman and Ross (J. Chem. Soc. (C):199–203, 1969) gave a diisopropilidene derivative ($[M]^+$ at m/z 630 in EI-mass spectrum); hence arabinose is attached at C-3 and C-8 of flavonoid (I) since this isopropilidenation is specific for *C*-galactosyl and *C*-arabinosyl residues in mono- and di-*C*-glycosylflavones as described in the above review by Chopin *et al.* $FeCl_3$ oxidation of flavonoid (I) gave L-arabinose. The above results show that flavonoid (I) is 3,8-di-*C*-arabinosylluteolin (Fig. 1), a new natural product; this is the first report of a 3,8-di-*C*-glycosylflavone from ferns.

3,8-Di-*C*-glycosylflavones were found for the first time in plants in 1985 by Matsubara et al. (Nippon Nogeikayaku Kaishi 59: 405–410, 1985) who isolated apigenin 3,8-di-*C*-glucoside and diosmetin 3,8-di-*C*-glucoside from *Citrus sudachi* peelings; subsequently these two flavonoids have been found also in *Citrus sinensis* peelings (Agric. Biol. Chem. 50: 781–783, 1986) and the former flavonoid has been found also in *Citrus junos* peelings (Nippon Nogeikayaku Kaishi 59: 683–687, 1985) by Kumamoto et al.

Flavonoid (II) was identified as luteolin 6-*C*-arabinoside-8-*C*-glucoside by ultraviolet spectral analysis with usual shift reagents, treatment with 2N HCl (which failed to give an aglycone), ESMS (which gave a pseudomolecular

ion at m/z 603 ($[(M+H)+Na]^+$), $FeCl_3$ oxidation (which gave D-glucose and L-arabinose) and ^1H NMR spectrum (DMSO-d_6). Assignment of D-glucose to C-8 was based on doublings of signals in ^1H NMR spectrum (two signals were observed for H-3 and H-6′) since this feature is due to the presence of a *C*-linked hexose at C-8 as described in a review by Jay (pp. 57–93, *in* J.B. Harborne ed., *The Flavonoids, Advances in Research since 1986*, Chapman and Hall, London, 1994). Flavonoid (II) is a new fern constituent; it has previously been found in bryophytes (*Blepharostoma tricophyllum* and *Pleurozia conchifolia*) and in angiosperms (*Lespedeza capitata*, *Glycine max* and *Astrantia major*) as described in the review by Jay.

The author thanks MURST (Rome) for financial support. Mass spectral data were provided by SESMA (CNR, Naples).—FILIPPO IMPERATO, Dipartimento di Chimica, Università della Basilicata, I-85100 Potenza, Italy.

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Review

Pteridophytes of Upper Katanga (Democratic Republic of Congo), by Jan Kornaś, Anna Medwecka-Kornaś, Francois Malaisse and Malgorzata Matjaszkiewicz. 2000. Botanical Papers 35, Institute of Botany Jagiellonian University, Kracow, Poland.

This fairly standard pteridophyte flora covers the Upper Katanga, which is the southeastern part of the Democratic Republic of Congo (formerly Zaire). The area is bordered by Tanzania, Zambia and Angola in central Africa. The work is the outgrowth of collections by two Belgian botanists in Katanga as identified by the Polish pteridologist Jan Kornaś, author of many articles on African ferns. The paper was completed by Anna Medwecka-Kornaś after the death of Dr. Kornaś in 1994.

The 180-page book includes a description of the area (climate, geology, soils and vegetation), a list of species by family and genus (alphabetically), distribution maps, and remarks on the pteridophyte flora. The description of vegetation types is brief but complete, with correlation to soils and climate. There are, unfortunately, no keys or descriptions for the species; however, for each species, distribution, relative abundance, herbarium citations and habit are given. Taxonomic notes are useful for some species where there are questions or problems.

The final section of the work is a brief discussion of the taxonomic composition of the Pteridophytes to adjacent regions of Africa. The flora includes 183 species in 60 genera. *Asplenium* is the largest (27 species), as expected in this part of the world, followed by *Thelypteris* (12), *Selaginella* (11), and *Pteris* and *Trichomanes* (9 each). Although some species are relatively common, more than 50 species have been found at only one or two stations. Katanga has a richer pteridophyte flora than some other adjacent regions, but not as rich as tropical East Africa. The publication of this flora is important because the destruction of forests and political unrest in the region may prevent gathering of additional information in the near future.—James D. Montgomery, Ecology III, Inc., 804 Salem Blvd., Berwick, PA 18603.

American Fern Journal 92(3):248 (2002)

Review

The Illustrated Flora of Illinois. Ferns. 2nd ed., by Robert Mohlenbrock. Southern Illinois University Press, Carbondale. 240 pp. $39.95.

Local, illustrated fern floras provide a wonderful service by providing a handy, complete, and easy to use reference without the "chatter" of species not found in the area. These floras are designed to provide a streamlined, comprehensive view of the pteridoflora. Without a doubt, Mohlenbrock's first edition accomplished this goal and more. The Introduction provided a brief account of the history of Illinois fern collecting, a brief account of pteridophyte morphology, and a very nice discussion of habitats. Add to this the combination of functional keys, state maps, detailed species accounts, and excellent illustrations and you have a recipe for success. Having said this, the second edition is a considerable disappointment. All of the new material is packed into a 45 page Appendix. The new taxa are keyed out separately from all the rest in a section entitled "Key to the Additional Ferns of Illinois". For a plant not covered in the first edition, therefore, one must first attempt to identify the species in the main part of the book and then go to the Appendix and start over.

Much of the Appendix consists of a "do-it-yourself" editing experience, in which you look at information provided and then go into the main part of the text and fix it up. As an example, on page 180 you are told that the name for *Lycopodium porophilum* Lloyd & Underwood (see p. 24) is *Huperzia porophila* (Lloyd & Underwood) Holub and that you should add dots to the map on page 26 to include Brown, Carroll, Cumberland, JoDavies, Johnson, Lake, Lee, Randolph, Rock Island, Schuyler, and Will counties. To make things worse, for any genus in which new taxa have been added there are new keys that should be utilized, so please make note not to use the ones in the first 173 pages. The new illustrations while adequate for identification, in most cases lack the detail and precision of those found in the previous edition. While the second edition does provide up to date information, it seems unreasonable in this day of electronic wizardry that the changes found in the Appendix could not have been incorporated into a cohesive whole *before* publication.—R. James Hickey, Botany Department, Miami University, Oxford, Ohio 45056.

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Authors are encouraged to submit manuscripts pertinent to pteridology for publication in the *American Fern Journal*. Manuscripts should be sent to the Editor. Acceptance of papers for publication depends on merit as judged by two or more referees. Authors are encouraged to contribute toward publishing costs; however, the payment or non-payment of page charges will affect neither the acceptability of manuscripts nor the date of publication.

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AMERICAN FERN JOURNAL

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QUARTERLY JOURNAL OF THE AMERICAN FERN SOCIETY

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American Fern Journal 92(4):249–269 (2002)

The Morphological and Genetic Distinctness of *Botrychium minganense* and *B. crenulatum* as Assessed by Morphometric Analysis and RAPD Markers

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ABSTRACT.—Two species of *Botrychium* subgenus *Botrychium* (moonworts, Ophioglossaceae), *Botrychium minganense* Victorin and *B. crenulatum* W. H. Wagner, can sometimes be confused in the field, even by experts, because of their reduced morphology. *Botrychium minganense* can imitate *B. crenulatum*, which is more rare. They are afforded different degrees of protection on Federal lands, making the distinctness of these species a question of management, conservation, and systematic interest. The purpose of this study was to compare a morphometric analysis of these two species with an analysis of DNA markers from the same individuals, and to assess their distinctness under each method. Collections were made in Washington, Oregon, Idaho, and Montana from seven populations of *B. crenulatum* and 18 populations of *B. minganense*. Each plant was measured, emphasizing characters cited by authors in the original species descriptions. Canonical variate analysis performed on SAS separated the samples into two species groups with 32% overlap. RAPD genetic markers revealed more genetic variation than has previously been documented in moonworts. UPGMA cluster analysis of the similarity of RAPD profiles showed well-defined *B. minganense* and *B. crenulatum* clusters, but no distinct clusters within *B. minganense* that could be correlated with its morphological variability. Small samples of the moonwort species *B. lunaria* and *B. simplex* included for comparison also formed distinct clusters. *Botrychium crenulatum* had seven unique RAPD bands, and identification of *B. crenulatum* could be confirmed or ruled out with markers from one or two RAPD primers. Both *B. crenulatum* and *B. lunaria* have been suggested as possible diploid parents of tetraploid *B. minganense*. All RAPD markers absent in *B. crenulatum* but present in *B. minganense* were also present or polymorphic in *B. lunaria*, supporting *B. lunaria* as a possible parent. One very small population of *B. minganense* showed a monomorphic RAPD profile, consistent with inbreeding, but all other populations had multiple genotypes. Some plants of *B. minganense* clustered most closely with plants from populations up to 400 km away, suggesting that variation may be introduced into populations by occasional colonization by spores from distant sources.

Species of *Botrychium* subgenus *Botrychium* (moonworts, Ophioglossaceae) are an enigmatic part of the temperate flora, notable for their small size, reduced morphology and difficult identification. Several moonwort species in North America are listed as sensitive or rare because of small and/or few known populations. Small populations are more vulnerable to extirpation, whether from natural stochastic events or human activities. Understanding the threat to species requires accurate information on numbers of individuals and

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populations. If species intergrade morphologically, questions can arise not only about actual numbers of individuals and populations but also about species boundaries and the genetic distinctness of each species.

All moonworts are relatively small and bear a single leaf with a fertile segment (sporophore) and sterile segment (trophophore) each season from an underground bud. They are notoriously hard to find, especially in thick vegetation. As increasing emphasis has been focused on rare plants in recent decades, more concentrated searches have extended the known ranges of common *Botrychium* species and provided material from which 13 new species have been described since 1980. Several of these are endemic to western North America: *B. crenulatum* W. H. Wagner, *B. echo* W. H. Wagner, *B. lineare* W. H. Wagner, *B. montanum* W. H. Wagner, *B. paradoxum* W. H. Wagner, *B. pedunculosum* W. H. Wagner, *B. pumicola* Coville, and *B. pinnatum* H. St. John (the latter two described in 1900 and 1929, respectively). Distinguishing moonwort species in the field often depends on subtle differences in phenology, color, texture, proportions of the parts of the single leaf, and dissection of the pinnae. Such species, poorly morphologically differentiated but evolutionarily distinct, have been called cryptic species (Stebbins, 1950; Paris, Wagner, and Wagner, 1989; Hauk and Haufler, 1999). Although species differences may be subtle, some species are also quite variable among regions, among sites, and even within the same site (e.g., Wagner and Lord, 1956). One of those species is *B. minganense* Victorin.

This study was initiated in response to the practical need to distinguish between *B. crenulatum* and *B. minganense*. These two species have been confused in the western United States by many botanists (Zika, 1992). Although both have been listed as "sensitive" in the past by National Forests in the Pacific Northwest Region (Region 6) and the Northern Region (Region 1), *B. minganense* has been delisted in Region 6 in response to the discovery of many more populations, while *B. crenulatum* retains its official status as rare. Species designation affects management options where *B. crenulatum* occurs. The documented distribution of *B. crenulatum* is the mountain states of the American west (Arizona, California, Idaho, Montana, Oregon, Nevada, Utah, Washington, and Wyoming), whereas *B. minganense* is widespread in the western mountains and across northern North America (Wagner and Wagner, 1993).

Botanists have employed both lumping and splitting approaches to the confusing variability of *B. minganense*. *Botrychium minganense* has been interpreted by many authors as a variety of *B. lunaria* (L.) Sw. (see Wagner and Lord, 1956 for discussion). In *Flora of the Pacific Northwest* (Hitchcock and Cronquist, 1973), only five moonworts are recognized, and the taxon to which *B. minganense* keys is called *B. lunaria* var. *onongadense* (Underw.) House. Cronquist said that *B. minganense* is "...morphologically scarcely separable from diploid var. *onongadense*..." and considered it conspecific with *B. lunaria* (Gleason and Cronquist, 1991). *Botrychium minganense* is a currently accepted taxon (International Taxonomic Information System database http://www.itis.usda.gov/plantproj/itis, April 15, 2000; Kartesz, 1994). In the most

recent treatment of North American moonworts (Wagner and Wagner, 1993), *B. minganense* is reported to be sometimes misidentified as *B. dusenii* of South America. It is also easily confused with *B. lunaria* (Wagner and Lord, 1956; Farrar, 1998), *B. ascendens* (Zika, 1992; Farrar, 1998), *B. pallidum* (Zika, 1992), *B. spathulatum* (Zika, 1992), and *B. crenulatum* (Wagner and Lord, 1956; Lellinger, 1985; Wagner and Devine, 1989; Zika, 1992; Farrar, 1998). Zika (1992) described *B. minganense* as "treacherously variable". Just as *B. minganense* was recognized as an independent species from the more widespread and common *B. lunaria*, so too were *B. pallidum* and *B. spathulatum* formerly confused with *B. minganense*. Both Wagner and Wagner (1988), and Wagner (1994) have suggested that *B. minganense* may represent a species complex.

Unlike *B. minganense*, *B. crenulatum* is more constant in form when well developed, but as with any moonwort, the identity of small plants can be ambiguous. *Botrychium minganense* can approach the form of *B. crenulatum* closely. Wagner and Wagner (1981) state that some of the collections on which the original description of *B. crenulatum* was based were originally identified as *B. lunaria* var. *minganense*.

Botrychium crenulatum is diploid ($2n = 90$, F. S. Wagner, 1993), whereas *B. minganense* is tetraploid ($2n = 180$ Wagner and Lord, 1956; but see Hauk and Haufler, 1999). Many fern species, however, have races with different ploidy levels (e.g. *Asplenium trichomanes*, Wagner et al., 1993), and ideally, additional evidence of genetic differences would be employed to separate species (for discussion, see Gastony and Windham, 1989).

Molecular techniques are well suited to clarify problems of cryptic species. Hauk (1995) used *rbcL* sequences in a phylogenetic analysis of 20 species of *Botrychium* subgenus *Botrychium*. Hauk found that four samples of *B. minganense* (from Michigan, Colorado, and Ontario) shared identical sequences, along with *B. paradoxum* and *B.* ×*watertonense*, and lacked the single synapomorphies that distinguished the simplex and campestre subclades of the "simplex-campestre" clade. *Botrychium crenulatum* formed a separate clade with *B. lunaria*, identical in sequence to the United States *B. lunaria* sample, and well separated from the "simplex-campestre" clade by a total of nine substitutions.

In contrast to the *rbcL* data, which did not distinguish *B. crenulatum* from *B. lunaria*, isozymes differentiated *B. crenulatum* from all others (Farrar, 1998; Hauk and Haufler, 1999). Among the sampled diploids *B. crenulatum* was most similar to *B. lunaria*, but their genetic identity (Nei, 1978) was only 0.53 (Hauk and Haufler, 1999). *Botrychium minganense* possessed the highest variability of the western moonworts (Hauk and Haufler 1999, Farrar 1998), but neither study inferred the variation to be indicative of species-level differentiation within *B. minganense*.

Random Amplified Polymorphic DNA (RAPD) markers, a type of genetic fingerprint, have revealed a level of genetic variation useful for distinguishing populations and sometimes species, and typically possess more variation than isozymes (for reviews, see Bachmann, 1997; Crawford, 1997). RAPD has been particularly useful in assessing variation in rare plants because, as a PCR-based

technique, it requires only small tissue samples, fresh or dried. DNA markers such as RAPD may provide important information for critically assessing morphometric analyses in taxonomically confusing groups. Morphometric analysis can suffer from a circular logic in which a taxon exhibits a certain range of morphologic variation because of assumptions made in the assignment of specimens to that taxon. Assigning specimens based on genetic markers can provide more robust morphometric insights, as has been shown in a variety of studies (e.g., Hardig et al., 2000).

Thus, the goals of this study are 1) to determine the genetic distinctness of *B. minganense* and *B. crenulatum*, on the basis of RAPD markers 2) to document patterns of genetic variation within *B. minganense* and *B. crenulatum*, on the basis of RAPD markers, and 3) to assess quantitatively the morphological differences between plants of *B. minganense* and *B. crenulatum* classified on the basis of genetic markers.

Methods

Collections.—Samples were collected from seven populations of *Botrychium crenulatum* and 18 populations of *B. minganense* in the states of Washington, Oregon, Idaho, and Montana (Table 1). Within this region populations were chosen to include a full range of habitats and geography. Plants with morphology intermediate between the two species were collected when found, and small plants were collected as well as large, well-developed ones to represent a full spectrum of the morphology found in each population. Plants were collected throughout the spatial extent of each site. Sample sizes are given in Table 1. In addition, two populations of *B. lunaria* and one of *B. simplex* E. Hitchcock (both subgenus *Botrychium*) were collected to provide a larger sample of species level molecular comparisons. The ecological associations of *B. minganense* and *B. crenulatum* were quite different in different parts of their ranges. *Botrychium crenulatum* in Washington is sometimes found in somewhat wetter and more open habitats than *B. minganense*, but the large populations sampled for this study were all growing under a *Thuja plicata*/mixed conifer canopy on subirrigated ground. By contrast, the Goofy Springs, Oregon, population was growing in heavy graminoid cover in an opening on seepy ground; the Stewart Creek, Montana, site was a wet mowed roadside and ditch; and at Lapover Ranch, Oregon (the one site on private land), *B. crenulatum* and *B. minganense* were growing together in grass cover under *Pinus contorta* with no spring evident. In Washington, *B. minganense* was found almost exclusively under riparian *Thuja plicata* stands with depauperate understory, but one sampled population (Mill Gate) came from an herbaceous mountain meadow. The association with *Thuja plicata* stands may be an artifact of the circumstance that moonworts have mainly been searched for in association with proposed timber sales. In Oregon, the sampled *B. minganense* populations were all under forest canopy open enough to support a luxuriant shrub and/or herb layer, except Dusty, which was from a wet meadow.

TABLE 1. Collection locations of *Botrychium* used in this study. Collections from some sites were segregated under more than one collection number. Where more than one analyzed species occurred at a single site, each species is listed as a population with an identifying letter (m = *B. minganense*, c = *B. crenulatum*, L = *B. lunaria*). Vouchers are deposited in ID.

Species	Site (no. of plants)	Site abbreviation	Location	Voucher
B. minganense	Watson Point (2)	Watson	OR, Wheeler Co.	Swartz 387
	Flowery Trail (6)	Flowery	WA, Stevens Co.	Swartz 393
	Kelsey Creek (6)	Kelsey	MT, Lincoln Co.	Swartz 394
	Rock Bottom (7)	Rock	ID, Boundary Co.	Swartz 398
	PWB Deer (7)	Deer	ID, Boundary Co.	Swartz 399
	Wenatchee Ford TSHE (7)	WenT	WA, Chelan Co.	Swartz 401A
	Wenatchee Ford RUPA (5)	WenR	WA, Chelan Co.	Swartz 401B
	Devil's Club Creek (7)	Devil	WA Chelan Co.	Swartz 402
	Mill Gate (17)	Mill, MillB	WA, Chelan Co.	Swartz 403, Swartz 453
	Aladdin 1 (6)	Aladdin1	WA, Stevens Co.	Swartz 414
	Bulldog Cabin (5)	Bulldog	WA, Stevens Co.	Swartz 420
	Poison Springs (7)	Poison	OR, Grant Co.	Swartz 425
	m Hodgson Creek (7)	mHodgson	WA, Ferry Co.	Swartz 466
	m Rd. #9576 (6)	mRd9576, ManleyX	WA, Ferry Co.	Swartz 468. Swartz 486
	m Manley Creek (15)	mManley	WA, Ferry Co.	Swartz 470
	La Grande 32 (6)	LaG32	OR, Union Co.	Swartz 504
	Shady Camp (7)	Shady	OR, Wallowa Co.	Swartz 506
	Dusty (6)	Dusty	OR, Union Co.	Swartz/ Riley 508. Swartz/ Yanskey 509
B. crenulatum	Goofy Spring (7)	Goofy	OR, Crook Co.	Swartz 388
	Stewart Creek (5)	Stewart	MT, Flathead Co.	Swartz 396
	Okanogan Cabin (7)	OKCabin	WA, Okanogan Co.	Swartz 404
	Aladdin Blowdown (7)	Aladdin	WA, Stevens Co.	Swartz 427
	Deadman Creek (5)	Deadman	WA, Ferry Co.	Swartz 445
	c Hodgson Creek (7)	cHodgson	WA, Ferry Co.	Swartz 467
	Lapover Ranch (10)	Lapover	OR, Wallowa Co.	Swartz 507
B. lunaria	L Rd. #9576 (5)	LRd9576	WA, Ferry Co.	Swartz 469
	L Manley Creek (7)	LManley	WA, Ferry Co.	Swartz 471
B. simplex	La Grande Meadow (5)	LGMead	OR, Union Co.	Swartz 505

Plants were collected by snipping them off at ground level to avoid disturbing the roots and the next year's below-ground bud. This procedure is not believed to have a significant negative impact on survival (Johnson-Groh and Farrar, 1996; Montgomery, 1990). Where possible, plants were collected after they had shed spores. Plants were pressed, individually numbered, color photocopied, and digitally imaged before grinding for DNA extraction.

The color photocopies are deposited in the University of Idaho Herbarium (ID) as facsimile vouchers, along with additional collections from the same populations.

Morphometric analysis.—As a quantitative approach to capturing morphological subtlety, a morphometric analysis of characters that can be scored from herbarium specimens was made. Characters cited by authors in the original species descriptions were used whenever possible. Some characters that are valuable to botanists in the field, such as color, texture, or folding of pinnae, could not be scored because they are distorted or destroyed by pressing and drying. Forty-one different measurements or ratios were recorded for each plant. Measurements were made using a Panasonic WV-CD20 video camera and Mocha image analysis software (SigmaScan Pro version 3.0 Jandel Scientific).

Analysis.—Canonical discriminant analysis identifies one or more canonical variables that are linear combinations of multiple measured characters. These canonical variables can show the greatest morphological differences between groups. Statistical calculations were performed using the SAS CANDISC procedure, SAS Release 6.11 (SAS Institute Inc.), on the same samples of *B. minganense* and *B. crenulatum* used for genetic analysis, excluding three plants that were browsed. Two very unusual plants of *Botrychium minganense* also were excluded from the morphological analysis. One was extremely large, and the other had only rudimentary peg-like pinnae. One plant (cLapover.09) was excluded because it displayed an additive RAPD profile, and thus was possibly a hybrid. Preliminary one-way ANOVA showed that the means of many characters were significantly different between the species at the alpha = 0.05 level, including the ratio of trophophore width to the width of its axis (reflecting the tendency of pinna margins to be decurrent on the rachis); average angle of the margins of the four basal-most pinnae (degree of fanning); ratio of the length of the space between the first two pinnae pairs to greatest pinna width (a measure of overlapping of pinnae); ratio of greatest pinna width to least pinna width; ratio of length to width of trophophore; ratio of length to width of sporophore; ratio of pinna width to length; length of the sporophore; average angle made by the four basal-most pinnae with the rachis; total height (ground level to tip of sporophore); length of trophophore; length of trophophore stalk; and length of gap between first two pinna pairs. Measurements of these characters are illustrated in Figure 1. All pinna measurements were made from the same pinna for each plant, one of the largest pair. In the largest plants the lowest pinnae are sometimes partly transformed into sporangial branches. In that case one of the largest untransformed pair was chosen. The ratios of 1) trophophore width: trophophore axis width and 2) maximum:minimum pinna width were log-transformed, and the length of sporophore was square root-transformed to bring them closer to a normal distribution. The distribution of all variables used was judged to be within the limits of robustness of the procedures (K. Steinhorst, pers. comm.). Variances were compared between species groups for each character to see that they were equal, or if not, the variance of the larger group did not exceed that of the

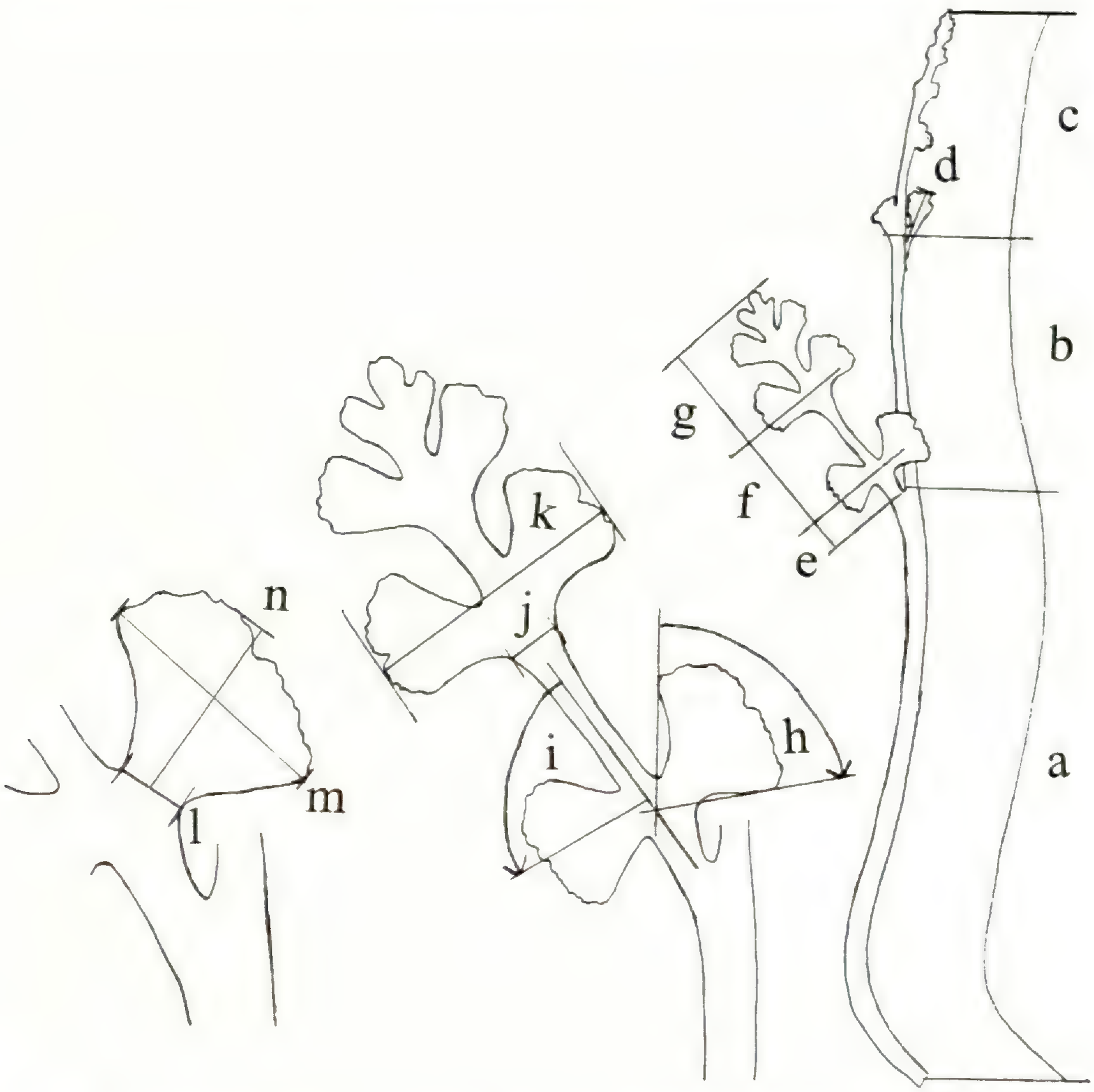

FIG. 1. Measurements made for characters that were significantly different between *Botrychium minganense* and *B. crenulatum* in morphometric analysis. a. Length of common stalk. b. Length of sporophore stalk. c. Length of sporangia-bearing part of sporophore (b+c = length of sporophore, a+b+c = total height. These and any other curved lengths were traced directly on the image of the plant). d. Length of longest sporophore branch (2d = sporophore width). e. Length of trophophore stalk. f. Distance between centers of first two pinna pairs. g. Balance of length of trophophore (e+f+g = length of trophophore). h. Angle of edges of pinna (for average of four basal-most pinnae). i. Angle at which pinna meets axis of rachis (for average of four basal-most pinnae). j. Greatest width of rachis. k. Trophophore width. l. Least width of largest pinna m. Greatest width of largest pinna. n. Length of largest pinna.

smaller group by more than a factor of 2.5, a conservative level chosen for unequal sample sizes. In general, variances were greater for *B. minganense*.

RAPD analysis.—DNA was isolated from 10 mg samples of each pressed plant. For those plants that were less than 10 mg, the whole plant was used. Plants were ground on ceramic well plates with liquid nitrogen, ground further

with 600 µl 70°C CTAB buffer, and transferred to 1.5 ml tubes. The grinding buffer and subsequent isolation procedures followed Stewart and Via (1993), with the following modifications: the homogenate was incubated at 70°C for 30 minutes before the chloroform extraction, the precipitated DNA pellet was washed with 1 ml cold 76% ethanol with 10 mM NH_4AC, and the dry pellet resuspended in 50 µl TE. Of several tested, this protocol was the least likely to yield gummy residues coprecipitating with the DNA, which was a problem with some samples. The residue, when it occurred, was removed by centrifugation before quantifying the DNA with a fluorometer. DNA was amplified (Williams et al., 1990) in 25 µl reactions containing 1× buffer (Promega M190A), 0.1 mM of each deoxynucleotide, 2 mM $MgCl_2$, 0.00005% bovine serum albumin, 5 pmols 10-mer primer (Operon), 10 ng genomic DNA, and 0.5 units *Taq* DNA polymerase (Promega), overlaid with 25 µl mineral oil. Samples were amplified in an MJ Research PT 100 thermocycler (44 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, with a final 5 min at 72°C). Products were electrophoresed in 1.5% agarose gels, visualized by UV illumination after staining with ethidium bromide, and imaged with AlphaImager v. 3.2 software. Populations were divided among multiple PCR runs, and a subsample was run multiple times to confirm repeatability of each band chosen. Bands were scored manually by comparison to standard size markers. Bands are designated by the name of the primer with the approximate size in base pairs as a subscript, e.g. B-11_{575}.

Primer screening.—Primers were screened against two samples each of *B. minganense* and *B. crenulatum*. Twelve primers (A-11, B-11, B-12, C-6, C-8, C-9, C-10, C-11, D-11, D-16, D-20, X-1) showing the best well-spaced bands polymorphic in one or both species were selected for the final data set. One hundred ninety-four plants were scored manually for presence or absence of 74 RAPD bands each. As more species and populations were added, fewer primers and bands within primers could be used because some new bands were close to the position of old bands or amplified with different intensity, making them difficult to score. Therefore, the scoring is conservative and reflects only minimum differences among all populations, whereas many additional differences that are not included in the data set are readily apparent among individuals and populations in the same gel.

Cluster analysis of RAPD data.—UPGMA cluster analysis was performed on RAPD data with NTSYSpc version 2.02 (Rohlf, 1997) using simple matching and Jaccard metrics.

Results

Morphometric analysis.—Optimal separation of the two species on a morphological basis requires consideration of multiple characters at once. The six variables in Table 2, when analyzed together, provided the greatest separation of the groups in this data set. The canonical variate analysis tested the null hypothesis that there are no differences between the two species based on the chosen variables. This hypothesis was rejected at the $p = 0.0001$ level, with

TABLE 2. Correlations and coefficients of the six variables that provided the greatest discrimination between *Botrychium minganense* and *B. crenulatum* in Canonical Discriminant Analysis.

Variable	Pooled Within Canonical Structure CAN1	Pooled Within-Class Canonical Coefficients CAN1
AVANGMAR	0.50	0.65
AVANGPIN	0.16	0.40
PWIDLEN	0.20	−0.18
MLSPORO	−0.18	−0.94
NTWIDAX	0.51	0.51
NPINMAMI	0.41	0.52

AVANGMAR=average angle of pinnae margins; AVANGPIN=average angle of pinnae with rachis; PWIDLEN=ratio of pinna width:pinna length; MLSPORO=square root transformed length of sporophore; NTWIDAX=log-transformed ratio of trophophore width greatest width of trophophore axis; NPINMAMI=log-transformed ratio of greatest pinna width:least pinna width.

F = 35.52 and degrees of freedom of numerator 6 and denominator 164. Canonical scores may be computed by taking the original value for each plant on each measurement, multiplying it by the respective canonical coefficient from CAN1 (Table 2), and adding all these products plus a constant adjustment for the means. Scores graphed by species form two overlapping groups, with the mean for *B. crenulatum* at 1.81 and the mean for *B. minganense* at −0.71 (Fig. 2). CAN1 scores of 55 of the total of 171 plants, or 32%, fell in the 0 to 2 range where species identity is ambiguous. Scores of 92 plants of *B. minganense* out of 123 (75%) fell in the 0 to −4 range, and 23 plants of *B. crenulatum* out of 48 (49%) scored from 2 to 4, where each had a high probability of correct species identity. Only one *B. minganense* had a CAN1 score above 2.

Canonical variates can be interpreted in terms of those variables that contribute the most to the separation of the groups. Although canonical variates are artificial and must be interpreted with caution, they can be identified in terms of their correlations with the original individual variables (Johnson and Wichern, 1992). These "within" structure coefficients indicate how closely a variable and the canonical variate are related, or the extent to which they carry the same information (Klecka, 1980). The ratio of trophophore width:trophophore axis width had the highest correlation, 0.51, followed by average angle of pinna margins, 0.50, and pinna maximum width:minimum width, 0.41. The within-class correlation for pinna width: length was 0.20, average angle of pinnae to rachis 0.16, and length of sporophore −0.18.

Another way of looking at the contributions of each individual variable within classes is by comparing coefficients that have been transformed so their standard deviations are equal to 1. These standardized coefficients then measure the relative contribution of each variable to the canonical variate score. As a relative measure, the standardized coefficient of each variable will change depending on the contribution of other variables. If two variables share

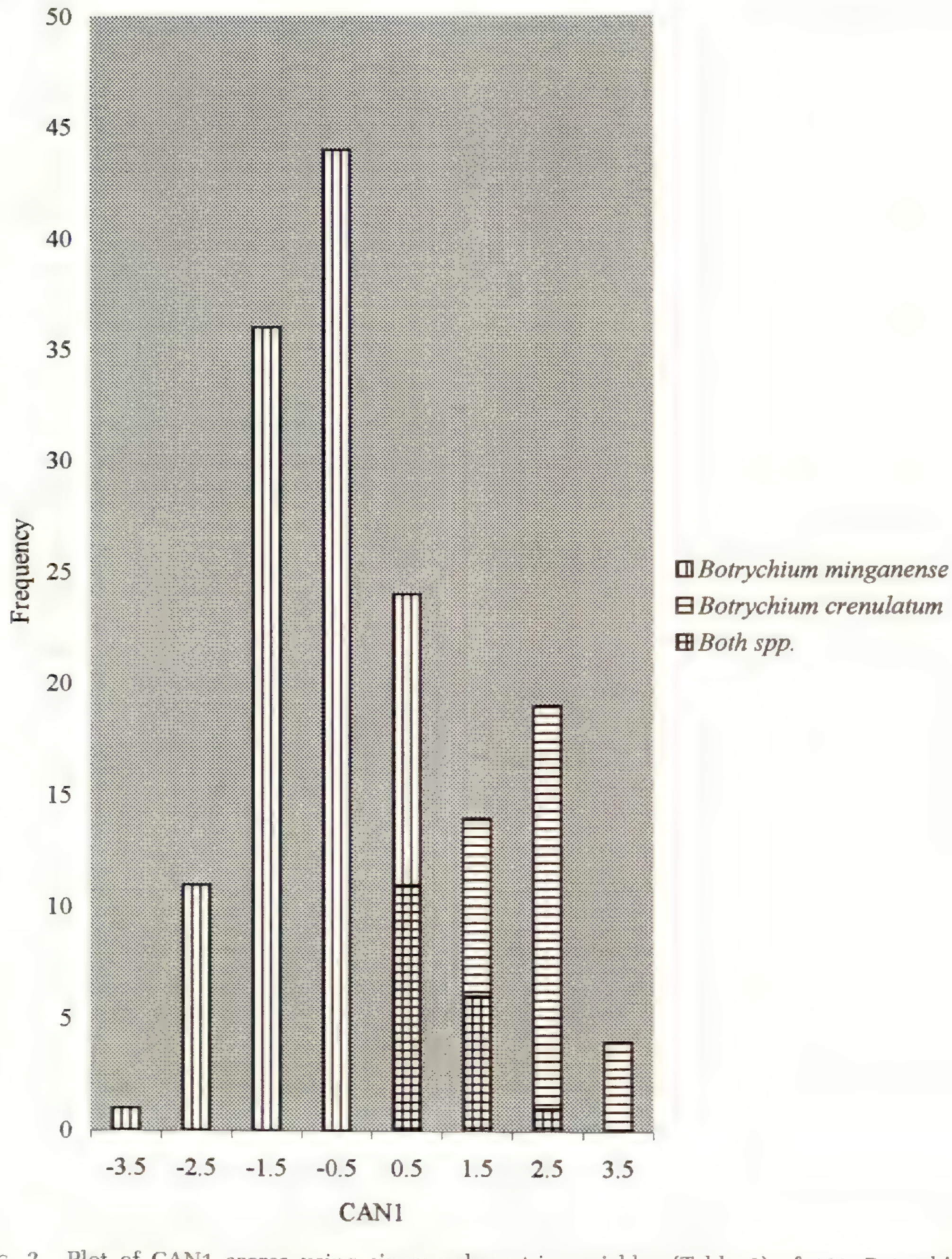

FIG. 2. Plot of CAN1 scores using six morphometric variables (Table 2) of 123 *Botrychium minganense* and 48 *B. crenulatum* plants whose species identity was confirmed genetically.

some of the same information (are highly correlated), the standardized coefficient value will be partly divided between them, but if one variable was not used, the standardized coefficient of the other would rise. They could also be larger but have opposite sign, so that one partially cancels out the other. The within structure coefficients, by contrast, are simple bivariate correlations

(Klecka, 1980). The standardized coefficient for length of sporophore, −0.94, had the highest absolute value among the variables. This was the measurement reflecting total size of the plant that contributed most to separation of the species. When it was included, other direct measures of size, such as total height, had small absolute values. Average angle of pinna margins, 0.65, and ratio of pinna maximum:minimum width, 0.52, are each related to the fanning of the pinnae in different ways, and they do not cancel each other out. The pooled within-class standardized canonical coefficient for ratio of trophophore width:trophophore axis width was also high, 0.51, and average angle of pinnae, 0.40, and ratio of pinna width:length, −0.18, were lower.

RAPD diagnostic bands.—*Botrychium crenulatum* was most differentiated (Table 3), set apart by seven bands (B-11_{1400}, C-6_{825}, C-8_{850}, C-8_{1700}, C-9_{1180}, C-10_{1275}, and D-11_{1075}) that did not occur in the other three species. One band (B-11_{1075}) was present in *B. crenulatum*, absent in *B. minganense* and *B. simplex*, and polymorphic in *B. lunaria*. One band (C-9_{1000}) was present in *B. crenulatum*, absent in *B. minganense*, and polymorphic in *B. lunaria* and *B. simplex*. Bands not present in *B. crenulatum* included six that were present in all individuals of *B. minganense*. Of these, three (C-11_{675}, D-16_{775}, and D-20_{890}) were polymorphic in *B. lunaria* and present in all *B. simplex*, two (B-11_{575}, C-9_{690}) were polymorphic in *B. lunaria* and absent in *B. simplex*, and one (D-11_{1225}) was present in *B. lunaria* and polymorphic in *B. simplex*. Four bands not present in *B. crenulatum* were present at high frequency (0.97–0.99) in *B. minganense*. Three of these (D-11_{875}, D-16_{400}, D-16_{510}) were polymorphic in *B. lunaria* and absent in *B. simplex*, and one (D-11_{1300}) was polymorphic in both *B. lunaria* and *B. simplex*. No bands in the sampled plants were unique to *B. minganense* or *B. lunaria*, and one band (C-6_{450}) was seen only in *B. simplex*. Bands common to all four species were not scored.

Clustering.—UPGMA clustering of the RAPD data using a simple matching metric resulted in four well-defined species groups (Fig. 3). The *B. crenulatum* cluster was most distinct. The *B. simplex* cluster and the *B. lunaria* cluster grouped together, and the "simplex lunaria" cluster associated most closely with the *B. minganense* cluster. Use of a Jaccard metric, discounting 0/0 matches, produced relationships conforming to those discussed below, except that the *B. lunaria* cluster associated most closely with the *B. minganense* cluster, and the *B. simplex* cluster grouped with the "minganense lunaria" cluster (dendrogram not shown). The *B. lunaria* and *B. simplex* clusters are displayed in Fig. 4.

Botrychium crenulatum.—Within the *B. crenulatum* group (Fig. 5), the largest cluster contained all the plants from Washington except two. This Washington cluster contained four subgroups within which plants had identical profiles. One subgroup included five OKCabin plants, and three contained plants from Deadman, cHodgson, and/or Aladdin. All *B. crenulatum* populations were polymorphic. Associated with the Washington cluster was a cluster that contained samples from Montana (Stewart) and Oregon (Lapover), plus two genetically distinct plants from the Hodgson population from northeastern Washington, which includes both *B. minganense* and

TABLE 3. Diagnostic RAPD bands.

Band name	*B. crenulatum*	*B. minganense*	*B. lunaria*	*B. simplex*
B-11$_{575}$‡#	0	1	P	0
B-11$_{1075}$**	1	0	P	0
B-11$_{1400}$***	1	0	0	0
C-6$_{450}$	0	0	0	1
C-6$_{825}$***	1	0	0	0
C-8$_{850}$***	1	0	0	0
C-8$_{1700}$***	1	0	0	0
C-9$_{690}$‡#	0	1	P	0
C-9$_{1000}$*	1	0	P	P
C-9$_{1180}$***	1	0	0	0
C-10$_{1275}$***	1	0	0	0
C-11$_{675}$‡▽	0[a]	1	P	1
D-11$_{800}$*	1	0	P	1
D-11$_{875}$†#	0	P(1)	P	0
D-11$_{1075}$***	1	0	0	0
D-11$_{1225}$‡○	0	1	1	P
D-11$_{1300}$†	0	P(1)	P	P
D-16$_{400}$†#	0[a]	P(1)	P	0
D-16$_{510}$†#	0	P(1)	P	0
D-16$_{775}$‡▽	0[a]	1	P(1)	1
D-20$_{890}$‡▽	0	1	P	1

1 = present, 0 = absent, P = polymorphic, P(1) = polymorphic, present very high frequency.
*** Present in *B. crenulatum*, absent in *B. minganense, B. lunaria, B. simplex.*
** Present in *B. crenulatum*, absent in *B. minganense*, polymorphic in *B. lunaria*, absent in *B. simplex.*
* Present in *B. crenulatum*, absent in *B. minganense*, polymorphic in *B. lunaria*, present or polymorphic in *B. simplex.*
‡ Present in *B. minganense*, absent in *B. crenulatum.*
† Polymorphic at high frequency in *B. minganense*, absent in *B. crenulatum.*
# Polymorphic in *B. lunaria*, absent in *B. simplex.*
▽ Polymorphic in B. lunaria, present in B. simplex.
○ Present in B. lunaria, polymorphic in B. simplex.
[a] Also present in Lapover9.

B. crenulatum. One of these individuals, "m"Hodgson.05, was classified in the field as *B. minganense*, but clearly groups genetically with *B. crenulatum*. The most distinct group was formed by Oregon plants. Goofy was the only population to form an exclusive cluster. Goofy and Lapover grouped together, but some members of Lapover also clustered with Stewart, the Montana population, in the mixed cluster. cLapover.09 was the most dissimilar member of the *B. crenulatum* cluster, and in fact displayed three bands otherwise found only in *B. minganense*, *B. lunaria*, or *B. simplex* (C-11$_{675}$, D-16$_{400}$, and D-16$_{775}$) as well as all the bands displayed only in *B. crenulatum.*

Botrychium minganense.—In the *B. minganense* group (Fig. 6), three populations formed exclusive clusters: Watson (Oregon), Poison (Oregon), and Devil (Washington). All others formed mixed groups. Thirteen plants from the Idaho Panhandle and neighboring Montana populations (Rock, Deer, and Kelsey) had identical profiles. Some members of each of those populations

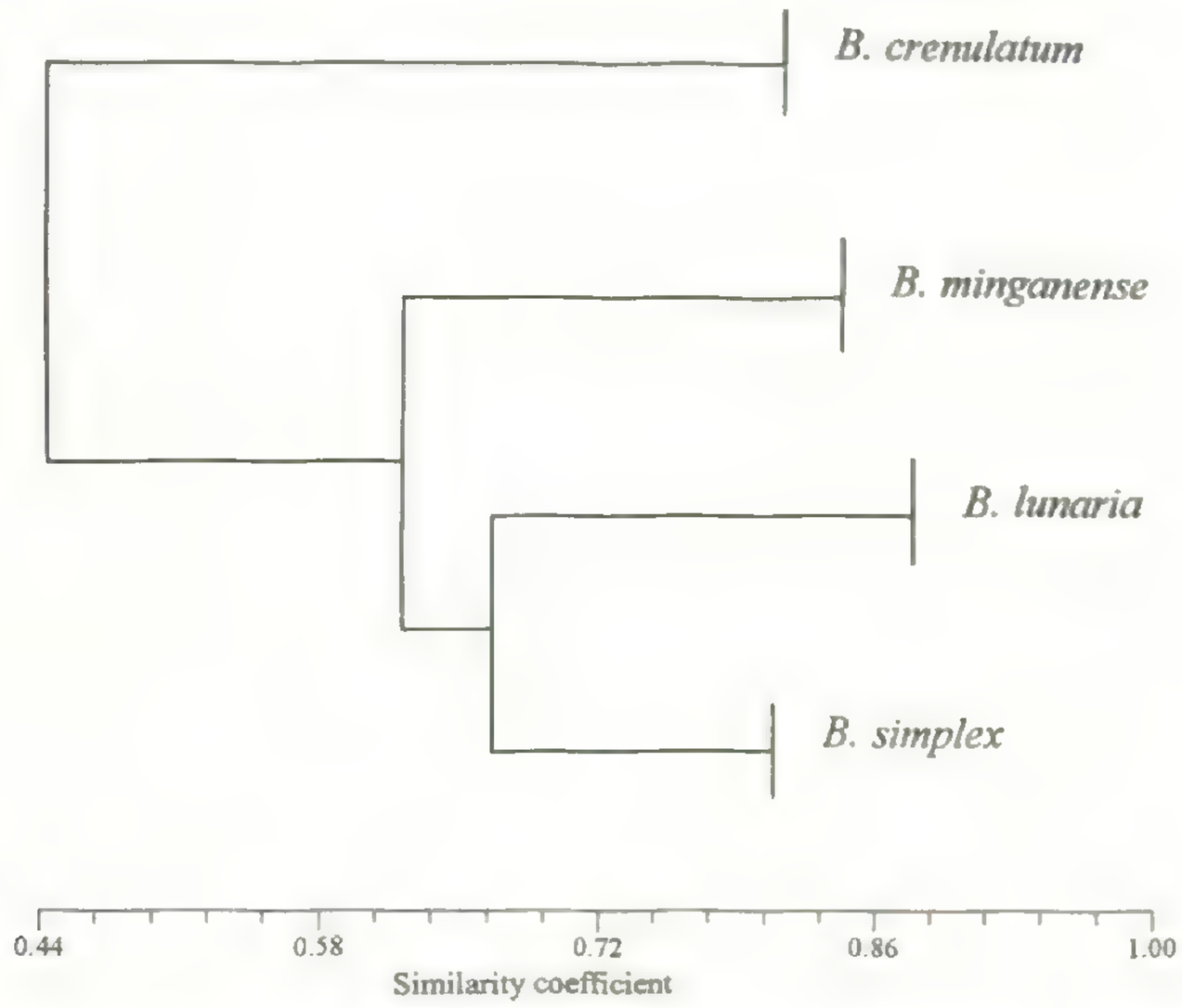

FIG. 3. Species clusters from UPGMA dendrogram of RAPD data from a total of 194 plants of four moonwort species: 49 *Botrychium crenulatum*, 128 *B. minganense*, 12 *B. lunaria*, and six *B. simplex*. The scale represents the similarity coefficient between clusters.

clustered with other groups. One population, Aladdin1, was monomorphic. Members of WenR and WenT, which grew adjacent to each other, each grouped in a separate larger cluster. Mill, from the Washington Cascades, and Manley, from northeastern Washington, were particularly diverse: each had members in four different larger clusters, and other members that were highly divergent.

DISCUSSION

Morphometrics.—The cryptic moonwort species *Botrychium minganense* and *B. crenulatum* can be separated by canonical variate analysis into two partially overlapping groups. Plotting the CAN1 scores of 171 plants whose identity had been genetically confirmed showed that 32% fell in the zone of overlap where the two species could not be separated using the characters scored. The characters that contributed most to the separation were measures related to pinna shape, proportions of the trophophore, and size. Pinna fanning, as reflected in the pinna shape characters, is emphasized in descriptions of *B. crenulatum* (Wagner and Wagner, 1981; W. H. Wagner, 1993). Average size of *B. minganense* is larger than that of *B. crenulatum* (mean height of sampled plants 84 mm and 73 mm respectively), but each can be less than a centimeter tall (pers. obs.). The ratio, width of trophophore: maximum width of trophophore axis, is a complex character that combines elements of several differences between the species, including length of pinnae, angle at which the pinnae meet the axis, and tendency of pinnae to be decurrent on the trophophore axis or of the axis to be flattened. These characteristics have not been emphasized in taxonomic descriptions of *B. minganense* and

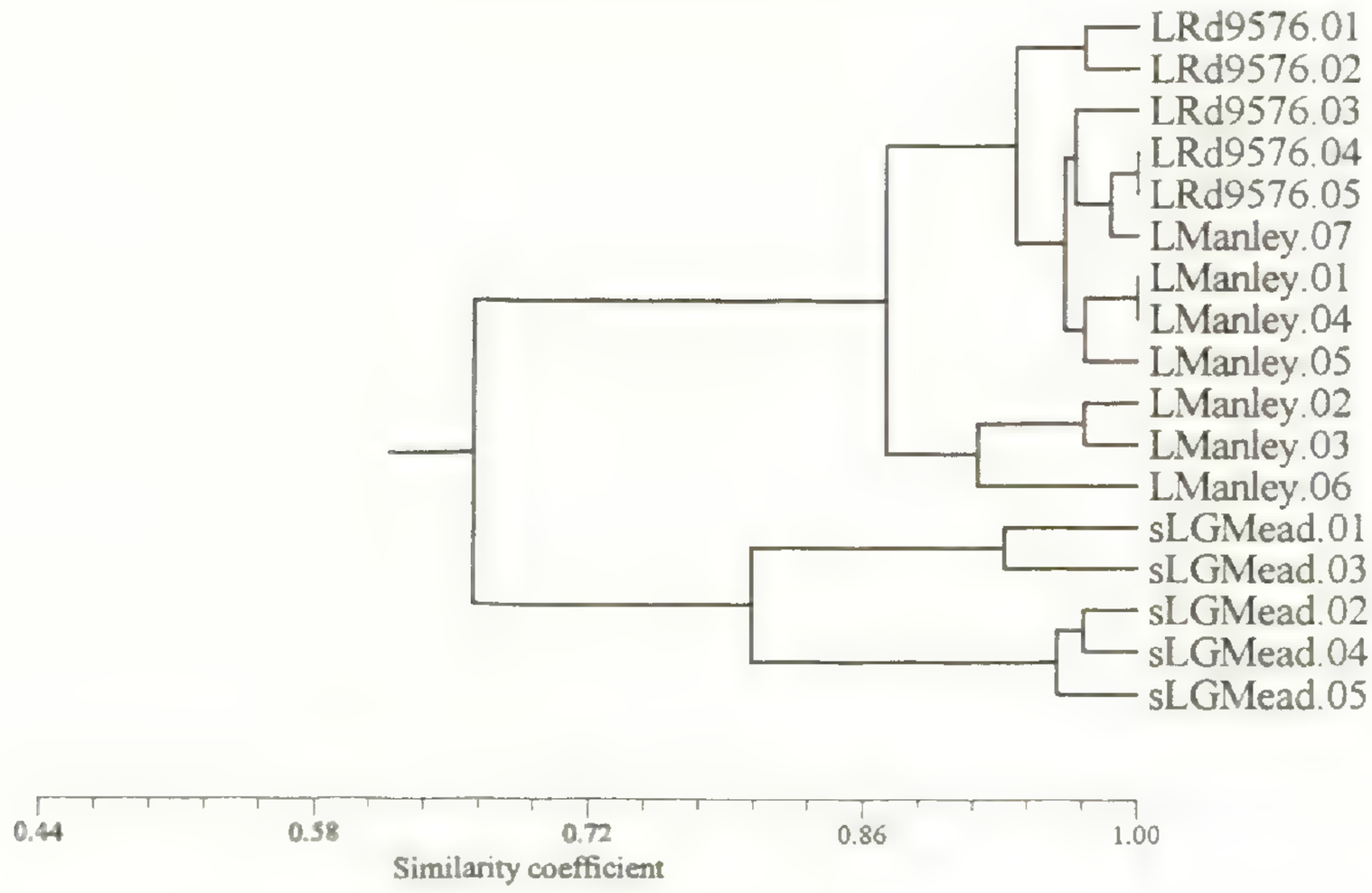

FIG. 4. Detail of *Botrychium lunaria* and *B. simplex* clusters from UPGMA dendrogram of RAPD data (Fig. 2). Plants are labeled by population abbreviation (Table 1) and individual number within population. Leading letter signifies species (L = *B. lunaria*, s = *B. simplex*). The scale represents the similarity coefficient between clusters.

B. crenulatum, although the trophophore of *B. minganense* has been described as narrow (Wagner and Wagner, 1993).

The statistical analysis of morphology was limited compared to field identification. Some useful morphological characters of live plants could not be used in an analysis of herbarium specimens. The characters that could not be captured include phenology, color, texture, and many aspects of plant habit, such as cupping of the pinnae. Other useful characters, such as the number of pinna pairs, or number of crenulations on pinna margins, are not normally distributed and therefore violate the requirements for canonical variate analysis.

The 32% ambiguity rate in the morphological analysis contrasts with the correct field identification of all but seven of the 171 analyzed plants. However, in the field, individual plants are not independently identified, as is the case with the statistical analysis. Field botanists generally examine the range of variation at a site and make an identification on the basis of a group of typical plants. Some well-developed plants will show characters that smaller ones lack. This is also true of herbarium identification. In fact, consulting botanists request collections of about a dozen plants for identification of moonwort species (Wagner, 1992; Wagner and Wagner, 1993; Zika et al., 1995). This study generally supports the assumption that similar plants associated at one site belong to the same species. In the genetic analysis, none of the populations identified in the field as containing *B. minganense* and not *B. crenulatum*, or *B. crenulatum* and not *B. minganense*, contained sampled individuals of the other species. However, even experienced botanists can occasionally be misled (example given in Farrar, 1998). Although we sought mixed populations for this study, only three of 23 (Hodgson, Manley, Lapover) had mixed *B. minganense* and *B. crenulatum* populations.

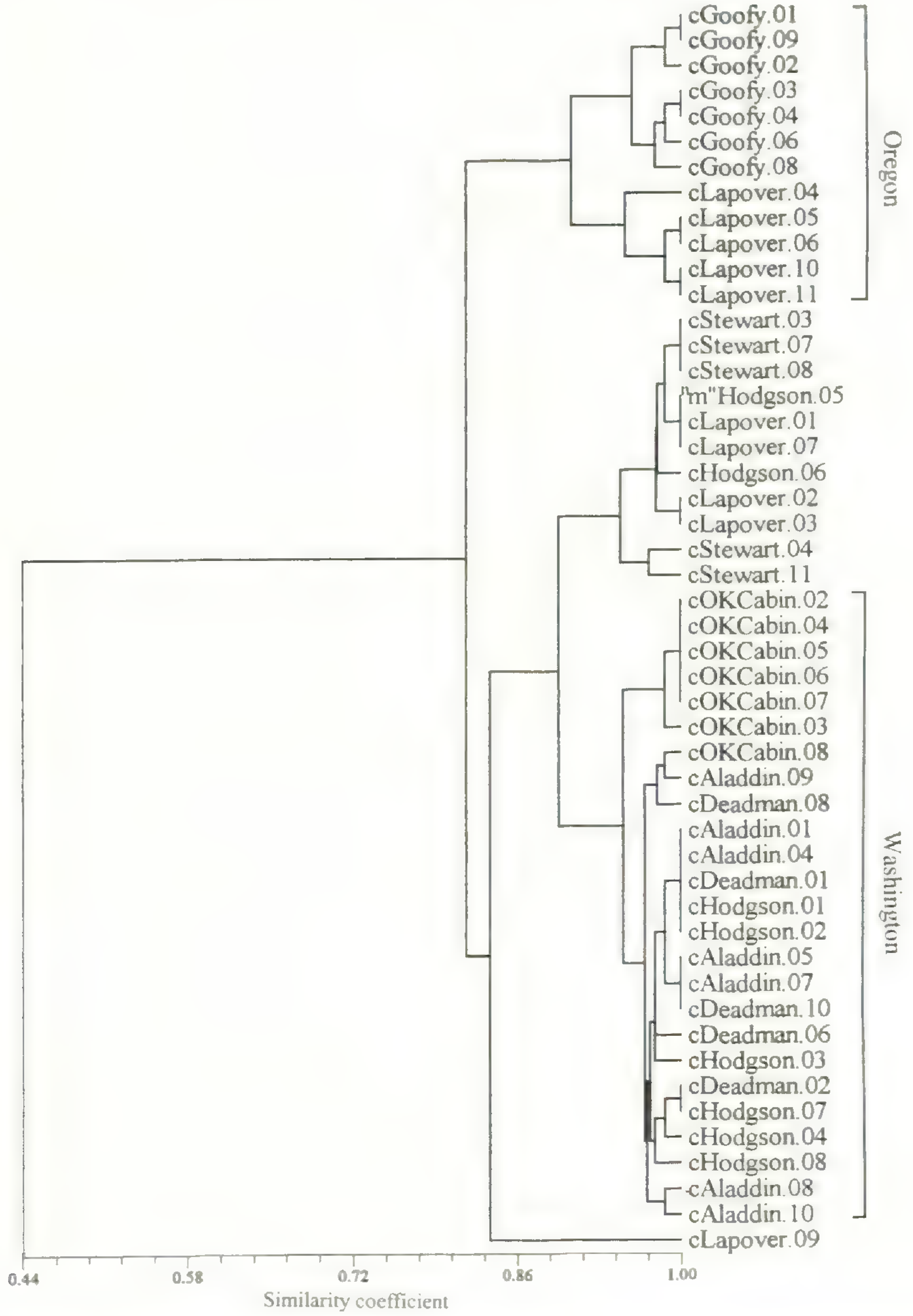

FIG. 5. Detail of *Botrychium crenulatum* cluster from UPGMA dendrogram of RAPD data (Fig. 3). Plants are labeled by population abbreviation (Table 1) and individual number within population. Leading letter signifies species as identified in the field (c = *B. crenulatum*, "m" = *B. minganense* as identified in the field). The scale represents the similarity coefficient between clusters.

RAPD analysis: Interspecific variation.—In contrast to the morphometric results, all sampled plants, with one exception (cLapover.09), grouped clearly by species based on RAPD markers. Primers C-10 and/or D-11, run with a known *B. crenulatum* sample, would be sufficient to confirm or rule out the identification of *B. crenulatum*. Separation of *B. minganense* from *B. lunaria* by means of RAPD markers requires more primers; because neither has unique bands and they are separated in the similarity analysis by differences in

FIG. 6. Detail of *Botrychium minganense* cluster (c) and subclusters (a, b) from UPGMA dendrogram of RAPD data (Fig. 3). Plants are labeled by population abbreviation (Table 1) and individual number within population. Leading letter signifies species as identified in the field (m = *B. minganense*). The scale represents the similarity coefficient between clusters.

frequencies of bands. All *B. simplex* sampled had one band that appeared to be unique to the species, but more populations must be sampled to verify this as a species marker. The assignment of plants to species based on RAPD markers agreed with their classification in the field, with one exception. That plant, "m"Hodgson.05, had a CAN1 score of 0.88 in the morphometric analysis (Fig. 2) and fell in the range of minganense/crenulatum overlap. It was field-identified as *B. minganense*, but had the RAPD pattern of *B. crenulatum*. It was a small plant with non-crenulated pinnae, from a mixed population of *B. minganense* and *B. crenulatum*.

RAPD analysis: Distribution of variation within species.—RAPDs provide greater evidence of variability in species of *Botrychium* than isozymes have. Hauk and Haufler (1999) reported 14 isozyme genotypes among 252 plants of *B. minganense*, and one genotype among nine plants of *B. crenulatum*. In this study, there were 100 RAPD genotypes among 128 individuals of *B. minganense*, and 28 genotypes among 48 plants of *B. crenulatum*. Within five populations of *B. minganense* (Watson, Bulldog, mHodgson, LaGrande32, and Dusty) and one of *B. crenulatum* (Deadman), no two sampled individuals had the same RAPD profile.

The population showing the highest genetic similarity among individuals was *B. minganense*–Aladdin1. This small population of about ten plants growing in approximately 9 m^2, was sampled heavily because it was morphologically ambiguous, and could not be identified to species in the field (W. H. Wagner, Jr., pers. comm.). The plants were small, light green, and displayed rather broadly fanned pinnae. Three of the sampled plants had CAN1 scores in the 0 to 2 range where the scores of species groups overlapped, and three had slightly negative scores in the "minganense" range. This was the only population that lacked within-population variation (scorable or unscorable) on the gels, and might be described as clone-like. All other sampled *Botrychium* populations were larger, and had more than one RAPD profile.

Most individuals from the Deer (Idaho), Rock (Idaho), and Kelsey (Montana) populations of *B. minganense* had the same RAPD profile. These populations were located within approximately 50 km of each other. However, proximity was not a good predictor of genetic similarity across all populations. The twin *B. minganense* populations from Wenatchee Ford, in Washington, grew about 30 m apart and were reported as one population on the Wenatchee Forest sensitive plant sighting form. They were kept separate in the analysis because one group was growing in deep shade in a riparian zone under *Thuja plicata* and *Tsuga heterophylla*, and the other was under *Rubus parviflorus* on a roadside. The *Tsuga* group clustered with the Mill population from a mountain meadow on the Wenatchee Forest, but the *Rubus* group clustered with Flowery and Bulldog, shaded forest sites from northeastern Washington, approximately 225 km away. No association between ecological sites and RAPD genotype is evident in this or other clusters.

Although some populations, such as Goofy (*B. crenulatum*) and Devil (*B. minganense*), showed low similarity to any other, most populations had members in more than one cluster. For example, some *B. minganense* plants

from Mill clustered with plants from Manley in northeastern Washington, whereas others clustered with Shady and LaGrande32 in northeastern Oregon, about 400 km from the Mill site.

Genetic variation within *B. minganense* did not suggest any coherent genetic groups that might be associated with its morphological variability. Hauk and Haufler (1999) reported more isozyme variability within *B. minganense* than any other polyploid sampled. Given that RAPDs are revealing more variability than isozymes, additional sampling from across the species range may reveal genetic patterns within the species.

Genetic structure of populations.—The contrasting patterns of genetic similarity may result from processes that hinder or promote genetic isolation. Two important factors influencing the structure of genetic variation in plants are breeding system and dispersal of propagules. The breeding system of moonworts is not known from experimental investigations, because they have not been cultivated successfully. The most direct evidence comes from the allozyme work of Hauk and Haufler (1999) on other species of subgenus *Botrychium*. Low variability within populations hampered their inferences of breeding systems, but they attributed the low frequency of heterozygotes found in four populations of diploid moonworts (*B. simplex* and *B. lanceolatum*) to inbreeding. Electrophoretic studies on *Botrychium* species in subgenus *Sceptridium* (McCauley et al., 1985; Watano and Sahashi, 1992) and subgenus *Osmundopteris* (Soltis and Soltis, 1986) reported extremely high levels of inbreeding. Outcrossing may be hindered by the underground gametophytes of this genus (Tryon and Tryon, 1982), although moonwort hybrids have been reported (Ahlenslager and Lesica, 1996; Wagner, 1980, 1991; Wagner et al., 1984; Wagner, Wagner, and Beitel, 1985; Wagner and Wagner, 1988), demonstrating at least occasional outcrossing. The number of allopolyploids also documents that outcrossing is an important evolutionary process in subgenus *Botrychium* (Hauk and Haufler 1999). In our study, the lack of genetic diversity in the RAPD profiles of the small Aladdin1 population is consistent with inbreeding.

Genetic variability within populations of an inbreeding species could be increased by immigration of propagules from distant sources, and occasional outcrossing. Fern spores are light and can travel long distances, as ferns colonize remote islands (Tryon, 1970; Tryon, 1986; Ranker et al., 1994). Tryon (1970) presented evidence that 800 km is not a significant barrier to the migration of a fern flora. Tryon and Tryon (1982) characterized Ophioglossaceae in particular as a colonizing group.

Because of the dominant inheritance of RAPD markers (Bachmann, 1997; Crawford, 1997), these data do not provide unequivocal insights into the breeding system of moonworts. Although the variability detected in this study may not have been predicted based on isozyme studies, it is not inconsistent with a high dispersal rate and a largely inbreeding mating system.

Ancestry of B. minganense.—The *rbcL* sequence of tetraploid *B. minganense* did not match that of any known diploid (Hauk, 1995). On the basis of the match between the hypothetical isozyme profile of the non-chloroplast parent

of *B. minganense* and the isozyme profile of *B. crenulatum*, Hauk and Haufler (1999), proposed *B. crenulatum* as the most likely candidate for that parent. The RAPD data, however, did not support this relationship, because *B. crenulatum* showed seven unique bands absent in *B. minganense*. An earlier hypothesis (F. S. Wagner, 1993) based on morphological data, proposed *B. lunaria* and *B. pallidum* as the parental diploids. The RAPD evidence is consistent with a close relationship between *B. minganense* and *B. lunaria*, because neither had bands that did not occur in the other. More genetic evidence is needed to clarify the origins of *B. minganense*.

cLapover.09.—The identity of one plant from the Lapover site in the Lostine River Valley, Oregon, was uncertain when it was collected. It combined the color and luster of *B. crenulatum* with rounded, broad-based pinnae otherwise seen only in *B. minganense*. The RAPD profile of this plant included all seven diagnostic *B. crenulatum* bands, plus three characteristic *B. minganense* bands including C-11_{675} and D-16_{775} (also polymorphic in *B. lunaria* and present in *B. simplex*), and D-16_{400} (polymorphic in *B. lunaria* and not present in *B. simplex*). Eight bands documented in all sampled *B. minganense* were not present in the plant. cLapover.09 appears to be a hybrid, both because of intermediate morphology and mixed markers. The three "minganense" markers could also have come from *B. lunaria* or *B. simplex*, but these species were not recorded from the site, whereas *B. minganense* and *B. crenulatum* were present. Other moonwort species recorded at the site were *B. ascendens* and *B. lineare*, for which we have no RAPD data. Neither of these moonworts typically has rounded pinnae. Because not all of the diagnostic *B. minganense* bands were present, cLapover.09 does not appear to be an F_1 hybrid between *B. minganense* and *B. crenulatum*, but is more likely a backcross or later generation hybrid derivative.

Conclusions

Although many plants of *B. minganense* and *B. crenulatum* could not be reliably distinguished by canonical variate analysis of morphology, all sampled plants, except an apparent hybrid, could readily be assigned to species on the basis of RAPD profile. This supports the distinctness of the two species although their morphologies intergrade.

Although breeding system cannot be inferred from dominant markers such as RAPDs, higher levels of variability were detected within populations and species than might be predicted from previous genetic data, which suggested a high level of inbreeding. Thus codominant DNA markers such as microsatellites might be a productive avenue for further research into breeding system and evolutionary processes in *Botrychium*.

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Reproductive Behavior of Cloned Gametophytes of *Pteridium aquilinum* (L.) Kuhn.

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ABSTRACT.—Spores from single fronds of three different taxa of *Pteridium aquilinum* (L.) Kuhn were collected at different sites in Scotland, England and Sri Lanka. Gametophytes developed from these spores were treated to produce arrays of genetically identical clones. Sporophyte formation was determined when such clones from the same or different gametophytes, derived from the same frond, were combined in pairs in all possible ways to produce a diallel mating scheme. A recurring pattern of presence or absence of sporophyte formation indicated the occurrence of two genetic classes defined by no or very few sporophytes in pairs within either class but high frequency production in pair combinations of clones from different classes. The usual failure of sister clone pairs to produce a sporophyte contrasts with the frequently high incidence of sporophyte formation on the part of single, isolated non-cloned gametophytes. This conflict of evidence is discussed in relation to genetic incompatibility or, alternatively, the control of antheridia formation. The genetic differences revealed in cloned gametophytes provide an empirical way of determining whether a given stand of bracken is made up of more than one individual.

The breeding behavior of bracken gametophytes presents some unresolved problems. Thus, Wilkie (1956) produced experimental evidence for genetic incompatibility in bracken by recording the frequency of sporophyte formation in combinations of clones from different gametophytes derived from single fronds. The clones were produced by harvesting the prothalli which proliferated from the margins of sectioned gametophytes. The results, in clones prepared from three Scottish populations of bracken, could be plausibly reconciled with the occurrence of two mating types in each population. Since combinations of clones between populations were cross-compatible, it appeared that a single locus, multi-allele system was present so that each sporophyte would be heterozygous for dissimilar alleles. It was also noted that, although well defined, the apparent incompatibility was not absolute since a low, variable frequency of sporophytes occurred among putatively incompatible combinations.

On the other hand, Klekowski (1972) reported that single, isolated gametophytes of bracken from different localities display wide variation in sporophyte formation, ranging from zero to nearly 100%, with most samples from different sites exceeding 30%. Self-fertilization in such gametophytes is the rule and incompatibility is conspicuously absent. The differences in frequency of sporophyte formation per sample were attributed to differences in the frequency of recessive sporophytic lethals for which the parent plants were heterozygous. He also noted that Wilkie's findings could be explained if the populations concerned carried balanced lethals, whereby each parent plant would be a double heterozygote for recessive sporophytic lethals at two loci linked in repulsion. Haploid gametophytes would carry one or the other of the

lethals and thereby present the appearance of two mating-types. If the lethality of either homozygous combination were incomplete i.e., the lethals were leaky, apparent cases of breakdown in the incompatibility system, inferred by Wilkie (1956), could be accounted for. Klekowski also indicated the need for further study of Scottish populations that might prove atypical, a suggestion that prompted the present study.

The experiments described here were designed to discover whether the appearance of two "mating-types", whatever their origin, could be detected in bracken populations from Scotland, England and Sri Lanka. It is particularly important to discover whether or not evidence from the British and Sri Lankan populations, geographically separated and belonging to different subspecies, leads to the same conclusions.

Material and Methods

Sample Sites.—Seventeen spore samples were obtained from single fronds collected at the sites indicated in Table 1. Ten Scottish spore collections were obtained from Clunie Dam (CD1 to CD5), Black Hill (BH3 and BH11), Temple (T1), Rubery Reservoir (R1) and Edgelaw Reservoir (E1). Spores were also obtained from one English site and two Sri Lankan sites: Farr's Inn and Bambarakanda Falls. Five samples (SL1 to SL5) were collected at the former and one (SL6) at the latter site.

Three taxa are included in these collections. Both *Pteridium aquilinum* (L.) Kuhn ssp. *aquilinum* and ssp. *fulvum* (Kuhn) Page & Mill. are included in the Clunie Dam samples. CD2, CD3, and CD4 belong to ssp. *fulvum* and CD1, and CD5 to ssp. *aquilinum*. The stand of ssp. *fulvum* is roughly triangular with sides of approximately 20 m (Page and Mill, 1994). Three fronds CD2, CD3, and CD4 were collected approximately 10 m apart from the west side of the stand. The ssp. *aquilinum* fronds, CD1 and CD5 were collected adjacent to, respectively, the north and south sides of the stand of ssp. *fulvum*, which is surrounded by sporophytes belonging to ssp. *aquilinum*. The Black Hill site refers to a roughly circular, isolated stand of ssp. *aquilinum* surrounded by *Calluna* moor. Two fronds were collected 60 m apart. The English population was from a scattered distribution of *aquilinum*.

From the Sri Lankan populations of ssp. *revolutum* (Kuhn) Wu Zheng-yi & Raven, which is common in upland areas and the only subspecies in the island, five fronds were collected over a 15 m distance within a fairly continuous stand bordering one side of a road. The other site, (SL6) is several miles away from Farr's Inn and at a lower elevation by some 1500 m.

Culture of Gametophytes.—Spores were collected overnight by inverting fertile fronds on paper. The spores were washed three times by centrifugation in sterile water. Single drops of suspended spores were transferred by micro-pipette to petri dishes with 1% agar (Sigma A7002) made up with Knop's solution and sterile water (Wilkie, 1956). All cultures were kept at 20°C under a standard fluorescent strip light, except for occasional periods under daylight

TABLE 1. Locations of the different single-frond spore collections from three sub-species of *Pteridium aquilinum* (L.) Kuhn. Nomenclature for British samples follows Page (1982) and Page and Mill, (1994), and for the Sri Lankan samples Wu Zheng-yi and Raven (1999). Map references for British samples refer to the U.K. Ordnance Survey, Landranger Series.

Site	Identification of spore collections	Map reference
ssp. *aquilinum*		
Clunie Dam	CD1, CD5	GR NN 915 592
Black Hill	BH3, BH1	GR NT 184 636
Rubery Reservoir	R1	GR NT 311 571
Edgelaw Reservoir	E1	GR NT 306 581
Temple	T1	GR NT 312 583
Hutton-le-Hole	Y1	GR SE 700 890
ssp. *fulvum*		
Clunie Dam	CD2, CD3, CD4	GR NN 915 592
ssp. *revolutum*		
Farr's Inn	SL1, SL2, SL3, SL4, SL5	80 E 49 6 E 49
Bambarakanda Falls	SL6	80 E 51 6 N 46

and ambient temperature, which applied equally to all cultures within a set of comparisons.

The frequency of sporophyte formation by gametophytes was recorded under the following conditions. When thalli had grown to about 0.5 cm diameter, a random sample was transferred individually to small compartments, 2 cm square and 1.7 cm deep, in plastic boxes made up of 25 such compartments, each provided with sterile, washed sand moistened with Knop's solution. The appearance of a sporophyte was attributed to self-fertilization. Pairs of such gametophytes, whose members were from different sites, were also kept under similar conditions. Any sporophytes which appeared in the latter comparisons may have arisen by selfing or crossing between gametophytes.

A different kind of experiment was carried out with cloned gametophytes derived from single thalli. This entailed the combining of the cloned gametophytes in pairs, either according to a regular scheme described below, or randomly combined within or between different spore samples.

To produce clones, young spore derived gametophytes were either treated for five minutes with 0.5 M KCl (Dyer, 1979) and then washed with water or they were cut into segments. Most, but not all, gametophytes treated either way and kept thereafter on Knop's agar substrate produced many small thalli around the margins. These small thalli were removed and grown to produce arrays of genetically identical clones. For each sample of spores from a given collection, 25 randomly chosen gametophytes were used to produce clones. Either method of producing clones led to the same conclusions. Treated gametophytes differed in the rate of formation of daughter clones. When twenty or more clones became available, for at least nine or ten treated gametophytes, the clones were removed to set up the experiments described

TABLE 2. Diallel combinations of cloned gametophytes. The numbers 2 and 4 refer to the potential maximum number of sporophytes for combinations of clones from, respectively, the same or a different gametophyte. Combinations between identical clones occur once, but twice between clones from different gametophytes.

Clone numbers										
1	2	3	4	5	6	7	8	9	10	
2	4	4	4	4	4	4	4	4	4	1
	2	4	4	4	4	4	4	4	4	2
		2	4	4	4	4	4	4	4	3
			2	4	4	4	4	4	4	4
				2	4	4	4	4	4	5
					2	4	4	4	4	6
						2	4	4	4	7
							2	4	4	8
								2	4	9
									2	10

below. In the case of CD4, for reasons explained later, a particular test was repeated with sets of clones that had developed later.

To compare the behavior of cloned gametophytes they were transferred in appropriate pairs, when about 0.5 cm in diameter, to individual compartments of the plastic boxes under the conditions noted above. After ten to fourteen days they were irrigated with aerated tap water. This was repeated at intervals until two to three weeks had elapsed without the further appearance of sporophytes, when the experiment was terminated. The presence of sporophytes was determined by inspection with a low-power binocular microscope. To avoid possible damage due to handling and to avoid the risk of contamination, the occurrence of archegonia and antheridia was not followed during these tests. As will be apparent later, that in no way detracts from the significance of the evidence but points to an obvious subject of future enquiry.

ANALYTICAL PROCEDURE.—Genetic incompatibility has been claimed to account for the reproductive behavior of cloned gametophytes. An obvious way to check this is to set up a N × N diallel mating system whereby members of the arrays of clones derived from the same frond are combined in pairs in all possible ways, including combinations between sister clones. This results in the mating scheme illustrated in Table 2. For convenience it can be collapsed into the indicated triangular form. The lower diagonal position (diagonal slots) refers to the pairing of sister clones while all the other positions refer to combinations between the arrays (e.g., 1 × 2, 2 × 1). Since a gametophyte, cloned or otherwise, has the capacity to produce a single sporophyte, the maximum number of sporophytes expected in the diagonal slots is two whereas four are expected for all the other, duplicate combinations shown in the diagram. Interpretation of the reproductive behavior of clones depends on the nature of the departure from the numerical distribution shown in Table 2 when they are combined in such a diallel scheme.

It is first necessary to consider how the presence of a simple genetic incompatibility system would affect the distribution of sporophytes in a diallel test. In the case of two, equally frequent haploid mating types (+ and −) where only the heterozygote (+/−) will give rise to a sporophyte, the results of combining gametophytes from a heterozygous individual can be represented as:

	+	−
+	0	2
−	2	0

or, more succinctly as

	+	−
+	0	4
−		0

Extending the same sort of diagrammatic representation to the simplest hypothesis of diallel combinations in which the gametophyte clones in individual arrays are all (+) or all (−), the results can be ordered to display the characteristic pattern shown in Table 3a. Note there is a rectangular set of positions, representing the heterozygotes, with a maximum number of four sporophytes. All the other positions will fall into one of the two triangles that represent either the (++) or the (−−) homozygotes; these do not produce sporophytes. In a diallel test of this kind the practical task is to see whether the order of the paired gametophytes can be arranged to display the characteristic pattern of sporophyte production.

Exactly the same kind of pattern can be generated if the parent individual is heterozygous for two recessive sporophytic lethal genes at two loci linked in repulsion, in which case only the double heterozygote will give rise to a sporophyte. This balanced lethal situation (Table 3b) leads to the same pattern of sporophyte production as the case of simple incompatibility, provided the linkage is complete. For either hypothesis the model assumes equal numbers of the alternative genotypes among the gametophytes which give rise to the clones used in any diallel test. In practice there will be chance variation about the 1:1 ratio and this will lead to corresponding departure from the precisely symmetrical pattern of the theoretical distribution illustrated in Tables 3a and 3b. Where it is necessary to test for departure from a 1:1 ratio the Chi-Square test has been used.

Results

Sporophyte Production in Non-cloned Gametophytes.—Young gametophytes were removed from the agar plate at random and allowed to develop either on their own (Table 4) or in the proximity of another gametophyte derived from a different frond of the same or a different taxon (Table 5). In the first situation the comparisons include samples from ssp. *aquilinum* (CD1, CD5, E1, R1, and T1), from ssp. *fulvum* (CD2, and CD4) and from ssp. *revolutum* (SL1, SL3, SL4, SL5, and SL6). Among these isolated gametophytes, the frequency of sporophyte production ranged from 0.16 to 0.76, with an average of 0.43. These data are consistent with the variation reported by Klekowski (1972). In the second design, the combination of gametophytes from different sources resulted in a much higher frequency of sporophyte production. Almost all (0.95) of a total of 598 such combinations produced at least one and often two

TABLE 3. The potential maximum numbers of sporophytes produced by crossing, in all possible ways, clones from eight different gametophytes derived from a sporophyte heterozygous for either: a) (+) and (−) genotypes or b) balanced lethals linked in repulsion. It is assumed that alternative haploid genotypes are equally frequent in each situation. The diagonals refer to the single combinations of identical clones; all other combinations occur twice.

a) gametophytes derived from a sporophyte heterozygous for (+) and (−) genotypes

Clone numbers and genotype									
1	2	3	4	5	6	7	8		
+	+	+	+	−	−	−	−		
0	0	0	0	4	4	4	4	+	1
	0	0	0	4	4	4	4	+	2
		0	0	4	4	4	4	+	3
			0	4	4	4	4	+	4
				0	0	0	0	−	5
					0	0	0	−	6
						0	0	−	7
							0	−	8

b) gametophytes derived from a sporophyte heterozygous for balanced lethals linked in repulsion

Clone numbers and genotype									
1	2	3	4	5	6	7	8		
+1	+1	+1	+1	1+	1+	1+	1+		
0	0	0	0	4	4	4	4	+1	1
	0	0	0	4	4	4	4	+1	2
		0	0	4	4	4	4	+1	3
			0	4	4	4	4	+1	4
				0	0	0	0	1+	5
					0	0	0	1+	6
						0	0	1+	7
							0	1+	8

sporophytes, whether the combinations were within or between taxa (Table 5). The lower production of sporophytes in isolates suggests a high incidence of recessive, sporophytic lethals at different loci. Given full penetrance of sporophytic lethals and heterozygosity in the source sporophytes for one, two, or three different, independently assorting lethals, frequencies of respectively 0.5, 0.25 and 0.125 are expected in random samples of isolated, selfed gametophytes. All the tests referred to in Table 4 can be reconciled with heterozygosity for either one or two lethals except T1 and SL5 in which the proportion of combinations with a sporophyte significantly exceeds 0.5 ($p < 0.01$). However, for environmental or genetic reasons, not all lethals may be fully expressed when homozygous. Lethals may be "leaky" so that sporopyhte occurrence is higher than would otherwise be predicted, possibly so in T1 and SL5. The high frequency of sporophyte production with gametophytes from different sources (Table 5) is also consistent with the occurrence of recessive lethals at different loci.

TABLE 4. Sporophyte frequency in single, isolated, non-cloned gametophytes derived directly from spores. N refers to the number and + and 0 to those with or without a sporophyte.

Origin	N	+	0	% fertile
ssp. *aquilinum*				
CD1	50	14	36	0.28
CD5	25	15	10	0.60
E1	99	50	49	0.51
R1	100	55	45	0.55
T1	99	66	33	0.67
ssp. *fulvum*				
CD2	50	12	38	0.24
CD4	25	16	9	0.64
ssp. *revolutum*				
SL1	25	7	18	0.28
SL3	25	5	20	0.20
SL4	25	4	21	0.16
SL5	25	19	6	0.76
SL6	25	7	18	0.28

CLONED GAMETOPHYTES OF SSP. *FULVUM*.—Tables 6 and 7 show the diallel combinations for the Clunie Dam samples derived from the stand of *fulvum* (CD2, CD3, and CD4). The original gametophytes used to produce clones were arbitrarily assigned numbers e.g., CD1 to CD25 to identify the clones derived from a particular gametophyte. These are the axes in Tables 6 to 9 and 11 to 13. For each analysis, the combinations have been arranged to best compare the observed distribution of sporophytes with the patterns illustrated in 3a and 3b of Table 3.

The distribution of sporophyte production in Table 6 for pair combinations can be explained by the presence of two genotypes for which the parent frond was heterozygous. When members of a gametophyte pair belong to the same genotype, sporophyte formation does not occur but does so when they differ in this respect. All sister clone pairs, derived from the same gametophyte (the diagonal slots), failed to produce sporophytes and the pattern of presence or absence of sporophytes corresponds to the pattern in both Tables 3a and 3b. Thus, for CD2 clone numbers 1, 4, 5, 6, 15, 2, and 9 did not produce a sporophyte or only rarely did when paired with a member of that set. Similar results are seen for the members of the other set, clone numbers 3, 10 and 14. However, when members of different sets were combined at least one and often three or four sporophytes were produced. Occasionally, one or two sporophytes occur where, according to either the incompatibility or balanced lethal model, none are predicted. The same pattern is encountered with CD3. The two categories or classes of clone include numbers 7, 9, 20 and 21 on the one hand, and numbers 4, 1, 9,12, 16 and 17 on the other, with the same qualifications as noted for CD2.

The diallel test with CD4 was carried out twice (Table 7). In the first test, performed at the same time as the tests with CD2 and CD3, all nine series of

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TABLE 5. Frequency of at least one sporophyte per pair (+) when non-cloned gametophytes from different locations are combined in pairs. In all the Clunie Dam (CD) pairs one gametophyte belongs to *aquilinum* and the other to *fulvum*. The Sri Lankan pairs were derived from putatively different individuals of *revolutum*, while in the remaining combinations (SL3 and BH11) one gametophyte belongs to *aquilinum* and the other to *revolutum*. N refers to the number of pairs.

Pair combinations	N	+	0	% fertile
ssps. *aquilinum* and *fulvum*				
CD1 and CD2	174	170	4	0.97
CD1 and CD3	100	85	15	0.85
CD5 and CD2	25	25	0	1.00
CD5 and CD4	124	122	2	0.98
ssps. *revolutum* and *revolutum*				
SL1 and SL3	25	25	0	1.00
SL1 and SL4	25	25	0	1.00
SL3 and SL5	25	22	3	0.88
SL3 and SL6	25	24	1	0.96
SL4 and SL6	25	22	3	0.88
ssps. *aquilinum* and *revolutum*				
BH11 and SL3	50	48	2	0.95
Total	598	568	30	0.95

clones failed to produce sporophytes or did so only very rarely. As noted above, although the model assumes two equally frequent classes among the gametophytes, derived from the single frond that gave rise to the clones, chance will cause variation about a 1:1 ratio in a random sample. However, it seems improbable, but not impossible, that nine gametophytes would belong to a single genotype.

The test with CD4 was repeated with a second series of clones which had developed later. This second test is in accord with the data from the previous CD2 and CD3 tests, suggesting that the first test with CD4 was non-representative. In the second test, one genotype included clone numbers 10, 14, 23, and 25 while the other included clone numbers 24, 1, 8, 11, 13, and 15. To identify which of the two genotypes is the one represented in the first test, clone numbers 18 and 19 of the first test were combined with all but one of the different clones used in the second test. Table 8 indicates that all the clones used in the first test belong to the same genotype as clone numbers 10, 14, 23, and 25 of the second test. Although unlikely, these data support a departure from a 1:1 ratio of genotypes among the cloned gametophytes of the first test.

It was noted earlier that CD2, CD3, and CD4 were derived from a single stand of ssp. *fulvum*. It is therefore of interest to ascertain the genetic comparability among them. Clones belonging to the alternative genotypes of respectively CD2 and CD3, CD2 and CD4, and CD3 and CD4 were combined in pairs. To compensate for the occasional shortage of replicates, clones not represented in the original test were included e.g., CD3 number 18 and CD4 number 3. One combination was lost due to algal infection. Table 9 indicates identity of the

TABLE 6. Diallel tests with the Clunie Dam samples (CD2 and CD3) of cloned gametophytes of ssp. *fulvum*. The clone numbers have been arranged to reveal the pattern of combinations that do or do not produce sporophytes, as in Tables 3a and 3b.

CD2 clone numbers										
1	4	5	6	15	2	9	3	10	14	
0	0	0	0	0	1	2	1	3	3	1
	0	0	0	0	1	1	3	4	4	4
		0	0	0	0	0	4	3	2	5
			0	0	0	0	3	3	2	6
				0	1	0	4	2	3	15
					0	1	4	3	4	2
						0	4	3	4	9
							0	0	0	3
								0	3	10
									0	14

CD3 clone numbers										
7	19	20	21	4	1	9	12	16	17	
0	0	1	2	2	2	3	3	3	3	7
	0	0	0	3	3	2	3	2	2	19
		0	0	1	2	2	2	4	4	20
			0	3	3	3	3	4	2	21
				0	0	0	0	0	0	4
					0	0	0	0	0	1
						0	1	0	0	9
							0	0	0	12
								0	0	16
									0	17

two genotypes in the three sets of cloned gametophytes. Pairings within genotype failed to produce sporophytes or did so only rarely so whereas pairing between genotypes often yielded the maximum of two sporophytes. By cross referencing, the total number of clones listed in the tests described in Tables 6 to 9 can be assigned to two genotypes comprising 24 and 16 samples respectively. This is not significantly different from a 1:1 ratio ($\chi^2 = 1.6$, $p > 0.1$). This evidence makes it likely that the stand of ssp. *fulvum*, from which CD2, CD3, and CD4 were collected, constitutes a single individual. This conclusion is also consistent with the results of randomly combined, paired, cloned gametophytes either from the same or different fronds from the stand of ssp. *fulvum* (Table 10, Sections i and ii). Among these pairs within fronds there is a 1:1 ratio of combinations that produce sporophytes and those that fail to do so. The same is generally true for combinations of clones derived from different fronds, except for a statistically significant excess of pairs which produce sporophytes when clones belonging to CD2 and CD3 were combined ($\chi^2 = 13.5$, $p < 0.01$). This is in sharp contrast to the combinations of cloned gametophytes between taxa, Sections iii and iv of Table 10. In these combinations, there is a consistently high incidence of sporophyte formation and often the maximum is produced.

TABLE 7. The repeat diallel test with the Clunie Dam sample (CD4).

CD4 i)

2	6	16	17	18	19	20	21	22	
0	0	1	0	0	0	0	0	1	2
	0	0	0	0	0	0	1	0	6
		0	0	0	0	0	0	0	16
			1	0	0	0	0	0	17
				0	0	1	0	0	18
					0	0	0	0	19
						0	0	0	20
							0	0	21
								0	22

CD4 ii)

10	14	23	25	24	1	8	11	13	15	
0	0	0	0	4	3	3	3	2	3	10
	0	0	0	3	4	3	3	3	4	14
		0	1	2	4	4	3	3	1	23
			0	4	3	4	2	4	1	25
				0	0	2	0	0	0	24
					0	0	0	0	0	1
						0	0	0	0	8
							0	0	0	11
								0	0	13
									0	15

CLONED GAMETOPHYTES OF SSP. *AQUILINUM*.—These experiments involve two samples collected adjacent to and on opposite sides of the stand of ssp. *fulvum*. Pairing among clones of either CD1 or CD5 were carried out in the same manner as described for *fulvum* and the results are shown in Table 11. In the CD1 diallel combinations the situation is similar to that already seen in *fulvum*. The diagonal slots are all zero. It appears that clone numbers 1, 3, 7, 11, 14 and possibly 12 belong to one genotype and 2, 5, 8, and 9 belong to the other. In CD5, although there is again evidence for the presence of two genotypes, there are more exceptions to the predicted occurrence of sporophytes. Thus, three of the ten pairings of sister clones give rise to one or two sporophytes. Clone numbers 2, 5, 13, 17, and 10 probably belong to one genotype and clone numbers 11, 12, 20, 4 and 6 to the other.

The two Black Hill samples (Table 12) also differ to some extent from each other. In BH3, clone numbers 1, 3 and 9 appear to belong to one category and numbers 4, 6, 12, 14, and 19 to the other; clone numbers 15 and 20 are exceptions. In their case sister clone pairing leads to the appearance of either one or two sporophytes in the diagonal slots. Also both numbers 15 and 20 produce sporophytes when paired with a member of either of the two sets: (1, 3, and 9) or (4, 6, 12, 14, and 19). In the balanced lethal model, this could occur if linkage is incomplete so that recombination in the parent sporophyte produces gametophytes which do not carry either of the sporophytic lethals. Alternatively, interactions with genes at other loci might be responsible for

TABLE 8. Test of genetic identity among the CD4 clones of *fulvum*. The effect of combining in pairs clone numbers 18 or 19 of the first test with the CD4 clones used in the second diallel test. Each combination was represented by a single pair so the maximum predicted number of sporophytes is two.

CD4 clones of second test									
10	14	23	25	1	8	11	13	15	CD4 clones of first test
0	0	0	0	2	2	1	2	2	18
0	0	0	0	2	1	1	1	2	19

allowing sporophytes to develop when they would not otherwise do so. BH11 behaves like *fulvum* with two categories of cloned gametophytes that distinguish, respectively, clone numbers 11, 21, 25, 22 and 18 from clone numbers 24, 14 and 9. In the small York sample with five sets of clones there is again evidence of two categories, numbers 4, 5 and 16 and numbers 1 and 22.

CLONED GAMETOPHYTES OF SSP. *REVOLUTUM*.—The diallel results for SL1, SL2, SL3, and SL4 are shown in Table 13. All the diagonal slots are empty, except for one exception in both SL2 and SL3, and there is the now familiar pattern of two alternative sets of clones. For SL1, one set or genotype includes clone numbers 3, 4, 5, 6, 8, and 10 and the other includes clone numbers 1 and 2. In SL3 the alternative sets comprise clone numbers 2, 3, 4, 6, 7, 8, 9, and 15, on the one hand, and clone numbers 1 and 5 on the other. In SL4 the alternative sets or genotypes are clone numbers 1, 3, and 4 and clone numbers 5, 2, 6, 7, 8, and 9. None of these distributions depart significantly departs from a 1:1 ratio.

SL2 is inconsistent and illustrates the kind of exception referred to in CD5 and BH3. Thus, six of the clones fall into two categories, numbers 3, 4, and 5 and numbers 1, 7, and 9. Clone numbers 2 and 8 are exceptions. These clones produce sporophytes when combined with a sister clone or a clone belonging to either set (Table 13).

DISCUSSION

Three features of these experiments are of particular significance. Firstly, with very few exceptions, sister clone pairs fail to produce sporophytes. Pooling the data over all tests, only 12 sporophytes formed out of a total of 118 pairs of sister clones. Such infertility is not due to loss of potential to develop the hermaphroditic condition on the part of cloned gametophytes since the pairing of such clones derived from different spores collected at different sites or from different taxa regularly led to a high and often maximum rate of sporophyte production (Table 10).

Secondly, the evidence from the diallel experiments indicates that clones derived from a single frond belong to one of two classes such that, sprorophyte formation depends generally on the joint presence of a cloned gametophyte from each class (Tables 6 to 9 and 11 to 13). It is assumed that the difference between classes is genetic.

TABLE 9. Tests of genetic identity among clones of the Clunie Dam samples of *fulvum* (CD2, CD3, and CD4). Cloned gametophytes are combined in pairs. One combination (1 × 16) was lost due to fungal infection.

CD2 clones										
14	10	3	15	9	6	5	4	1	2	
0	0	1	1	2	1	1	2	1	1	1
1	1	0	1	2	1	2	1	1	1	9
0	1	0	1	2	1	1	1	1	1	12
0	0	0	1	2	2	2	1	-	1	16
0	0	0	2	2	1	1	1	2	1	18
0	1	1	1	2	2	2	2	1	2	4
1	1	1	0	0	1	1	0	0	0	7
1	2	1	0	0	0	0	0	0	1	19
1	1	1	0	0	0	0	0	0	0	20
2	2	2	0	0	0	0	0	0	0	21

CD3 clones

CD2 clones										
14	10	3	15	9	6	5	4	1	2	
0	0	0	1	1	2	1	2	2	1	3
0	1	0	2	2	1	2	2	1	2	6
0	0	0	1	2	1	1	2	2	2	16
0	0	0	1	2	2	1	1	2	1	17
0	0	1	2	1	1	1	2	2	1	18
0	0	0	2	1	1	1	2	2	2	19
0	1	0	1	2	1	1	1	2	1	20
0	0	0	1	1	1	2	2	2	1	21
0	1	0	1	1	2	2	2	1	1	22

CD4 clones

CD3 clones										
4	7	19	20	21	1	9	12	16	17	
1	0	1	1	1	0	0	0	0	0	2
2	2	1	1	1	0	0	1	0	0	6
2	2	1	0	1	0	0	0	0	0	16
0	2	2	2	1	0	0	0	0	0	17
1	2	1	2	2	0	0	0	0	0	18
1	1	0	2	1	0	0	0	0	0	19
0	1	2	2	1	0	1	0	0	0	20
1	2	1	2	1	0	0	0	0	0	21
2	1	0	1	1	0	0	0	0	0	22

CD4 clones

Thirdly, when young, non-cloned gametophytes are isolated, self-fertilisation regularly occurs and the variable lack of complete fertility may be ascribed to the incidence of sporophytic lethals for which the parent plant is heterozygous (Table 4). Thus, cloned and non-cloned gametophytes appear to differ dramatically in their capacity for self fertilisation. It was this contrast that prompted Klekowski (1972) to invoke the hypothesis of balanced lethals and suggest the possibility of atypical behavior in the populations studied by Wilkie (1956). Since the behavior of the cloned gametophytes is essentially the same in samples belonging to different taxa, or derived from geographically

TABLE 10. Frequency of formation of at least one sporophyte in random combinations of pairs of cloned gametophytes derived from the same or different spore samples.

Samples	N	Sporophyte production +	−	Frequency
i. *fulvum*—Clunie Dam				
CD2	50	24	26	0.48
CD3	50	24	26	0.48
CD4	50	30	20	0.60
Total	150	78	72	0.52
ii. *fulvum*—Clunie Dam				
CD2 and CD3	50	38	12	0.76
CD2 and CD4	50	22	28	0.44
CD3 and CD4	49	28	21	0.56
Total	149	88	61	0.59
iii. *aquilinum* and *fulvum*—Clunie Dam				
CD5 and CD2	25	25	0	1.00
CD5 and CD4	25	24	1	0.96
CD1 and CD5	25	21	4	0.84
Total	75	69	5	0.92
iv. *aquilinum* and *revolutum*				
BH3 and SL4	50	48	2	0.96

remote sites, it is likely that it holds for the *Pteridium* complex generally under the conditions provided in these experiments. At first sight the appearance of multi-allelic incompatibility looks like the obvious interpretation of the results of the diallel tests and this interpretation certainly cannot be excluded, although it encounters the embarrassing evidence for self fertilisation on the part of isolated, single non-cloned gametophytes. If gametic incompatibility does account for the reproductive behavior of cloned gametophytes it appears necessary to infer that the process of cloning has altered physiology or development to uncover an incompatibility system which is not normally expressed in non-cloned gametophytes.

However, it is necessary to enquire whether the apparent contradiction in the behavior of cloned and non-cloned gametophytes might be resolved within the framework of what is known about the reproductive behavior of gametophytes. Näf (1958) concluded that gametophytes form antheridia in response to antheridogen secreted into the medium by other, more rapidly growing gametophytes. If this external stimulus is absent, antheridia do not form although archegonia do. Hence, if a gametophyte develops from a single spore in isolation, it will be unable to undergo self-fertilisation. This appears to hold generally although exceptions may occur, especially after long periods of isolation.

It may be assumed that a cloned gametophyte behaves the same way and similarly requires an external stimulus to produce antheridia. The minimum requirement is the presence of another gametophyte that can provide the

TABLE 11. Diallel tests on cloned gametophytes of Clunie Dam samples (CD1 and CD5) of *aquilinum*. One combination (1 × 7) was lost due to algal infection.

CD1 clones										
1	3	7	11	14	12	2	5	8	9	
0	0	-	0	0	2	2	3	2	4	1
	0	0	2	0	0	4	2	3	2	3
		0	0	0	2	1	4	1	4	7
			0	0	2	4	4	1	1	11
				0	0	3	2	2	2	14
					0	3	2	3	1	12
						0	0	0	0	2
							0	0	0	5
								0	1	8
									0	9

CD5 clones										
2	5	13	17	10	11	12	20	4	16	
0	0	0	1	1	2	3	3	1	1	2
	0	0	0	1	3	3	3	3	3	5
		0	0	2	3	4	4	3	4	13
			2	1	3	4	3	3	3	17
				0	2	3	3	4	3	10
					0	1	2	0	0	11
						1	1	0	2	12
							1	0	1	20
								0	2	4
									0	16

stimulus, but not any gametophyte can provide it. The diallel tests suggest that the stimulus is generally present only when the two gametophytes concerned are genetically different. Since antheridogen is accepted as the agent which induces antheridia formation, one might wonder whether, within a species, it constitutes a single chemical entity or might occur in different forms which can be recognized by a gametophyte as different from the form it secretes, in which case only exposure to a different form would allow antheridia formation and hence the possibility of sporophyte formation. An alternative model could be envisaged in which the antheridogen is constant but other compounds take over the role just suggested, except that they would be responsible for determining whether or not a gametophyte responded positively to the presence of antheridogen.

At least, this hypothesis may have the merit of resolving the discrepancy between the behavior of sister clones and isolated non-cloned gametophytes and removes the need to invoke balanced lethals. Although separated at an early age, it is likely that the latter have already been primed to produce antheridia via exposure to the chemical stimuli contributed by genetically different gametophytes on the agar plate.

A number of Wilkie's (1956) results can be accommodated within this general scheme. Thus single, isolated gametophytes, derived from single spores by micro-manipulation, only rarely gave rise to a sporophyte. When

TABLE 12. Diallel tests with cloned gametophytes of *aquilinum* (BH3, BH11, and Y1).

				BH3						
1	3	9	15	20	4	6	12	14	19	
0	0	0	2	2	3	3	3	3	1	1
	0	0	3	3	3	2	2	2	3	3
		0	2	2	2	2	2	3	3	9
			2	2	2	2	2	3	3	15
				1	4	2	2	4	3	20
					0	0	0	0	0	4
						0	0	1	0	6
							1	2	0	12
								0	1	14
									0	19

				BH11					
11	21	25	10	22	18	24	14	9	
0	0	0	0	0	0	3	4	3	11
	0	0	0	0	0	4	3	3	21
		0	0	0	0	4	3	3	25
			0	0	0	3	2	2	10
				0	0	3	3	4	22
					0	3	3	3	18
						0	1	0	24
							0	0	14
								0	9

		Y1			
4	5	16	1	22	
0	0	0	2	3	4
	0	0	4	2	5
		0	3	2	16
			0	1	1
				1	22

such gametophytes from the same frond were combined in pairs, only half of the combinations produced sporophytes, an observation which suggested a role for incompatibility, and which is also consistent with the diallel analyses and the results of randomly combining cloned gametophytes from different arrays derived from the same frond. The analysis of clone properties, in Wilkie's case, followed a procedure that differed from that used in the present experiments. The capacity to form a sporophyte was ascertained for single cloned gametophytes, when combined with sperm suspensions prepared from alternative clones derived from the same frond. Two classes of clone were detected and sporophyte formation was attributed to the union of genetically different gametes. However, it seems not unlikely that the antheridia inducing agent(s) will be included in such sperm suspensions, thereby inducing antheridia formation that would not otherwise occur and making self-

TABLE 13. Diallel tests with cloned gametophytes of *revolutum*.

SL1

3	4	5	6	8	10	1	2	
0	0	0	0	0	0	4	3	3
	0	0	0	0	0	3	3	4
		0	0	0	0	3	3	5
			0	0	0	4	1	6
				0	0	3	3	8
					0	3	3	10
						0	0	1
							0	2

SL2

3	4	5	1	7	9	2	8	
0	0	0	4	3	4	2	2	3
	0	0	3	4	4	4	2	4
		0	4	3	3	3	3	5
			0	0	0	1	4	1
				0	0	3	2	7
					0	1	4	9
						0	3	2
							2	8

SL3

2	3	4	6	7	8	9	15	1	5	
0	0	0	0	0	0	0	0	2	3	5
	0	0	0	0	0	0	0	1	3	3
		0	0	0	0	0	0	1	2	4
			0	0	0	0	0	4	2	6
				0	0	0	0	2	2	7
					0	0	0	3	2	8
						0	0	2	3	9
							0	4	4	15
								1	0	1
									0	5

SL3

1	3	4	5	2	6	7	8	9	
0	0	0	2	3	1	2	1	2	1
	0	0	2	3	2	1	3	2	3
		0	1	3	1	3	3	1	4
			0	0	0	0	0	0	5
				0	0	0	0	1	2
					0	0	0	0	6
						0	0	0	7
							0	0	8
								0	9

fertilization possible. Hence, the assumption of obligatory cross-fertilization is open to doubt. It would be informative to repeat the experiment, using also a fraction of the original sperm suspension from which sperm had been removed by filtration. To distinguish between self- and cross-fertilization requires genetic markers. It would be feasible to make such a distinction by comparing appropriate, variable DNA sequences in tissues of sporophytes and the gametophytes used in such experiments. There remains, however, a problem in Wilkie's report of the production of sperm by cloned gametophytes. According to the hypothesis developed here that would not be expected. One can only speculate whether the process of producing sperm suspensions introduces special conditions not encountered in the present experiments.

It was noted in the diallel tests, that there are occasional instances in which a cloned gametophyte can produce a sporophyte when combined with any clone of either of the two classes derived from a single frond. This suggests there are additional genetic or environmental factors that can influence antheridia formation or genetic incompatibility, if that is operating. It must also be asked whether the process of cloning might introduce reactions, apart from the speculative suggestion noted above, that would not occur in a normal, uncloned gametophyte. Dopp (1959) in his studies of antheridia and archegonia formation in *Pteridium*, noted that when thallus wings were sectioned, antheridia arose only behind a cut in the area furthest from the meristem which is responsible for a diffusible inhibitor of antheridia formation. It is conceivable that clones that develop from different regions of the original gametophyte may not be equivalent in their capacity to form antheridia and this could contribute to a reduction in sporophyte number below the maximum predicted in some instances. Such an effect would introduce a stochastic element that would tend to obscure underlying regularity in the recognition of two classes of clone per frond. If present, such an effect appears comparatively unimportant since the regularity is not obscured, whereas pairing of clones between taxa or sites, where it should equally apply, regularly results in high rates of sporophyte formation.

One might also enquire whether the evidence from the experiments described here is relevant to the natural history of gametophytes in the wild. Little information exists on this score, although Voeller and Weinberg (1969) have drawn attention to the natural scarcity of both gametophytes and young plants. If antheridia formation depends on genetically determined stimuli, of the kind suggested in the interpretation of the diallel tests, then the behavior of gametophytes in the wild will be density dependent. If their frequency is low, as seems to be often the case, then such gametophytes will behave in the same way as gametophytes isolated from the spore stage: self-fertilisation will be unlikely, or at least infrequent. At intermediate density, fertility will largely depend on the joint presence of genetically different classes responsible for reciprocal induction of antheridia formation and this will tend to promote variability. One might also wonder how far a similar system might apply more widely in ferns.

Clearly many problems remain for future study, and the present experiments, with their limited objectives cannot settle the issues raised. In a different context, there is an unexpected by-product of the diallel analysis. Systematic combination of cloned gametophytes in such a scheme provides an empirical way of determining whether a particular stand of bracken is made up of more than one individual. For example, analysis of the samples collected from different locations within the Clunie Dam stand of ssp. *fulvum* (Table 9) suggested that it comprised a single individual which had spread to occupy a substantial area.

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Thanks are due to Drs. C. N. Page and A. Dyer for general information about ferns and especially to Dr. Page for bringing the Clunie Dam situation to my notice and for collecting the fronds from which the spores were obtained. I am indebted to Dr. Dyer for critical comment on the manuscript and to Professor John Thomson for stimulating discussion.

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A New Population of Aleutian Shield Fern (*Polystichum aleuticum* C. Christens.) on Adak Island, Alaska

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ABSTRACT.—We report and describe a new population of the endangered Aleutian shield fern (*Polystichum aleuticum* C. Christens.) discovered on Mount Reed, Adak Island, Alaska. The new population is located at a lower elevation than the other known populations, placing the species' known elevational range between 338 m and 525 m. The discovery of this population is significant because it increases the total number of known populations and individuals for the species.

The Aleutian shield fern, *Polystichum aleuticum* C. Christens., is one of the most restricted and rare ferns in North America (Smith, 1985); it is listed as an endangered species (U.S. Department of Interior, 1988). In a circumpolar assessment of the rare vascular plants of the Arctic, the fern was classified according to the IUCN Red List threat categories as "endangered" (Talbot *et al.*, 1999). The species was first collected by W. J. Eyerdam, an assistant to Eric Hultén, in 1932 on Atka Island, one of the Andreonof Islands located in the center of the Aleutian Island chain, Alaska (Fig. 1). Eyerdam's holotype specimen (*W. J. Eyerdam 1086*, 5 July 1932) is accessioned at S; isotypes are accessioned at CAS, DS, and US. The species was first described by Christensen (1938). Attempts to relocate the original collection site on Atka in years subsequent to the species' description were unsuccessful (Smith and Davison, 1988). Then, in 1975, a population of the species was discovered, this time on the northeast arm of Mt. Reed, on Adak Island (Fig. 1), also of the Andreonof Islands of the Aleutian Island chain, Alaska (Smith, 1985). Several subsequent searches, performed from 1986 to 1988, on various islands of the Aleutian Island chain (Adak, Kagalaska, Atka and Attu; see Talbot *et al.*, 1995 for review) failed to locate additional populations. Finally, in 1988 and 1993, we discovered a second and third population, respectively, again on Mt. Reed (Talbot *et al.*, 1995). Three populations comprising approximately 117 individual fern clumps are known for the species (Tande, 1989; Talbot *et al.*, 1995).

In August 1999, genetic studies of *Polystichum aleuticum* were initiated to assess the species' relationship to *P. lachenense* (Hook.) Bedd. of Asia, as recommended in Talbot *et al.* (1995). While collecting samples as part of this genetic research, as well as ongoing systematic monitoring of the three known populations (Anderson, 1992), we discovered a fourth population of *P. aleuticum*, located approximately 142 m below the two populations found on

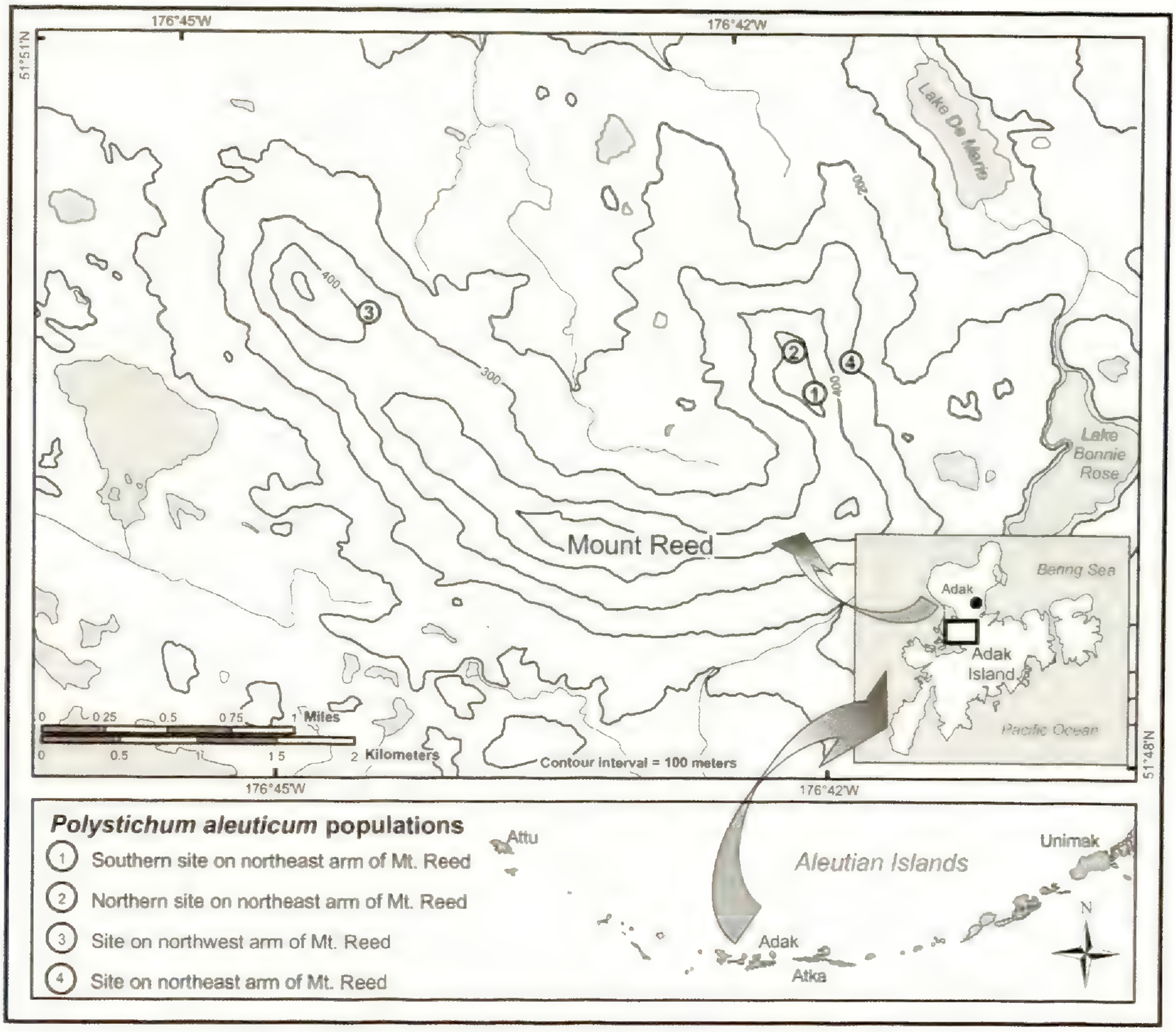

FIG. 1. Location of the four known populations of the Aleutian shield fern, *Polystichum aleuticum*, on Mt. Reed, Adak Island, Aleutian Islands, Alaska.

the northeast arm of Mt. Reed, Adak Island, at an elevation of 338 m. As was the case for the other three sites found on Mt. Reed, the fourth site was at the base of a steep rock outcrop on a northeast-facing slope. The slope angles at this site ranged from 60° to 90°. Notably, the site is located approximately 22 m below the lowest of the three previous populations (Population 3, Fig. 1, Table 1), in an area considered too treacherous to survey during earlier efforts due to steep, unstable, slippery slopes. This finding expands the elevational range of *P. aleuticum*, placing the species at elevations between 338 m and 525 m (Table 1). Also, notably, the fourth site is located on a northeast-facing slope. Climatologic records based on observations from 1950 to 1982 indicate wind direction on Adak is predominantly from the west-southwest, averaging 10.5 knots per hour, from June to November, the period of time during which the habitat would likely be free of snow (U.S. Department of the Navy, 1989). Thus, the occurrence of all known populations on northeast-facing

TABLE 1. Population characteristics of *Polystichum aleuticum* and associated geographical variables.

Island	Location	Pop. #	Latitude/ Longitude	Elevation (m)	Aspect	# Individuals	Year	Relocated
Atka	unknown, "within view of the village of Atka"[1]		unknown	unknown	unknown	unknown very rare	1932[1]	N
Adak	Mt. Reed, NE Arm	1	51° 49.640′ N 176° 41.861′ W	475.5–525.8	NE	98	1975[2]	Y
Adak	Mt. Reed, NE Arm	2	51° 49.491′ N 176° 41.776′ W	457.2–469.4	NE	14	1988[3]	Y
Adak	Mt. Reed, NW Arm	3	51° 49.960′ N 176° 44.141′ W	360.4	NE	5	1993[3]	Y
Adak	Mt. Reed, NE Arm	4	51° 49.378′ N 176° 41.733′ W	338.0	NE	14+	1999[4]	Y

[1] Christensen (1938).
[2] Smith (1985).
[3] Talbot *et al.* (1995).
[4] Present study.

slopes suggests habitats supporting populations of *P. aleuticum* are those offering protection from high winds during critical growth and reproductive periods.

At least 14 clumps constituted the new population; however, due to the inaccessibility of some of the vertical rock faces, a complete count of individual clumps was not possible. This number therefore underestimates the size of the population. Among the 14 clumps counted, five are associated with rock grottos; the remaining clumps are found on ledges on steep rock faces. Unlike the other locales, no clumps were associated with herb meadows. Clumps in the grottos and ledges comprise from five to 30 fronds, with all clumps containing fronds with sori. Clumps associated with steep rock faces comprise 12–40 fronds, again with all clumps examined containing fronds with sori. However, for safety reasons, not all clumps on vertical rock faces were examined to determine the number of fronds or presence of sori. The presence of sori on some *P. aleuticum* fronds suggests this population may be useful if spores were used for controlled propagation.

We recorded vascular plants associated with the new population; nomenclature follows USDA, NRCS (2001). Vascular plants associated with grotto and ledge clumps were the creeping dwarf shrub *Salix rotundifolia* Trautv.; the forbs *Achillea millefolium* L. var. *borealis* (Bong.) Farw., *Anemone narcissiflora* L., *Arnica unalaschcensis* Less., *Campanula lasiocarpa* Cham., *Conioselinum gmelinii* (Cham. & Schlecht.) Steud., *Huperzia chinensis* (Christ) Czern., *Lycopodium alpinum* L., *Pedicularis verticillata* L., *Platanthera sp.* L. C. Rich., *Polygonum viviparum* L., *Polystichum lonchitis* (L.) Roth, *Potentilla villosa* Pallas *ex* Pursh, and *Valeriana acutiloba* Rydb.; and the graminoids *Carex macrochaeta* C. A. Mey., *Carex circinata* C. A. Mey., *Poa sp.* (L.) Roth (viviparous) and *Tofieldia coccinea* Richards. Vascular plants associated with vertical rock faces included the forbs *Achillea millefolum* var. *borealis*, *Epilobium hornemannii* Reichenb., and *Viola langsdorfii* Fisch. *ex* Gingins; and the graminoids *Carex circinata*, *Poa sp.* (viviparous) and *Tofieldia coccinea*. Comparison of this list of associates from the other sites (Lipkin, 1985; Talbot *et al.*, 1995) indicates a high degree of similarity in species composition among the four known populations.

Using a hand-held Global Positioning System, we recorded precise geographic coordinates for this population as 51° 49.578′ N, 176° 41.733′ W. The discovery of this new population increases the number of known individuals to 131. We note here that this population was discovered accidentally while we were disoriented in the fog on Adak, and the area would, under normal circumstances, have been considered too treacherous to survey. It is very possible that additional populations of *P. aleuticum* inhabit Mt. Reed or nearby mountains, in areas too dangerous to survey without risk of injury, and we suggest the number of individuals representing *P. aleuticum* on Adak is likely underestimated, despite extensive surveys undertaken throughout the island from the mid-1980s to the mid-1990s.

This discovery is significant because it increases the number of known individuals by approximately 12%, and the number of known populations

from three to four, thus providing an increased buffer against loss of either individuals or populations of this rare and endangered species. It also expands the known elevational range within which future searches for new populations should be targeted.

Despite this new finding on Adak, however, *P. aleuticum* continues to be one of the rarest ferns in North America. While conducting botanical studies on other Aleutian islands between 1985 to 2001, the second author searched rocky outcrops similar to those on Adak that support the four known populations, without finding any new populations. These islands include: Adugak, Aiktak, Amlia, Buldir, Chagulak, Davidof, Kasatochi, Khvostof, Kiska, Nizki, and Uliaga islands. Additional surveys by both authors of Attu Island in 2000 and Simeonof Island of the Shumagin Island group, eastern Aleutians, in 1997 also failed to yield discoveries of new populations. Thus, biologists have searched for *P. aleuticum* on 16 islands in the Aleutian chain during the past fifteen years, and have found the fern only on the northern slopes of Mt. Reed, Adak Island.

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American Fern Journal 92(4):294–295 (2002)

Shorter Notes

***Trichomanes ribae* (Hymenophyllaceae), a New Filmy Fern from Costa Rica and Panama.**—The Hymenophyllaceae in Costa Rica and Panama are well known due to the works of Lellinger (Pteridologia 2: 185–228. 1989) and Pacheco (*in* G. Davidse, M. Sousa S., and S. Knapp, eds. *Flora Mesoamericana. vol. 1. Psilotaceae a Salviniaceae.* Univ. Nacional Autónoma de México, México, D. F. Pp. 62–83. 1995). However, as a result of additional studies during a recent trip to Costa Rica, a new species has been identified.

Trichomanes* (*Trichomanes*) *ribae Pacheco, *sp. nov.*—TYPE. Panama: Panama, 5–10 km NE of Altos de Pacora, on trail at end road, 750 m, 7 Mar 1975, *S. Mori & J. Kallunki 4964*, (holotype, MO). (Fig. 1)

Rhizoma repens, 0.1 cm diametro, trichomatibus catenatis; folia remota, 4.8–11.5 × 2.7–3.8 cm; petioli 0.08–0.5 × 0.05 cm, trichomatibus catenatis; laminae 4.7–11 cm lanceolatae, 2-pinnatifidae, apice pinnatifidae, basi subtruncatae; rachis alata; pinnae oblongo-lanceolatae, imbricatae; sori 1–4 per pinnam, involucris in lobis immersi, 0.2–0.25 × 0.2 cm, campanulatis, receptaculo exserto.

Rhizome long creeping, 0.1 cm in diameter with catenate trichomes; leaves distant, 4.8–11.5 × 2.7–3.8 cm; petioles 0.08–0.5 × 0.05 cm, nonalate, loosely and deciduously clothed with brown catenate trichomes, similar trichomes on rachis and veins; blade lanceolate, 4.7–8.3 cm, 2-pinnatifid, chartaceous, apex pinnatifid, base subtruncate; rhachis alate, wings 0.07–0.08 cm wide on either side; pinnae oblong-lanceolate, 11–18 on a side below the apex, 1.2–1.6 × 0.7, overlapping at in right angles to rhachis, their apices pinnatifid; segments oblong, 0.15–0.18 × 0.1–0.18 cm, apex obtuse to bifid, plane, margins complete; venation open, anadromous, pinnate, veins 2-furcate, not reaching the apices of the lobes; lamina cells almost isodiametric, translucent; sori lateral on the pinnae, 1–4 per pinna, involucres immersed, 0.2–0.25 × 0.2 cm campanulate, apex wide-flaring; receptacle exserted.

Paratype.—COSTA RICA: Limón; Siquirres, Las Brisas de margen izquierda de Quebrada Jesús, afluente innominado, Camino a Cerro Tigre. 09° 56′ 40″ N; 83° 25′ 15″ W, 800 m, 22 Mar. 1996, *G. Herrera 8849 & G. Valverde* (CR).

Trichomanes ribae belongs to subgenus *Trichomanes* as evidenced by the anadromous venation, sori lateral on the pinnae, distant leaves, and 2-pinnatifid lamina. Its nearest relative is *T. rupestre* (Raddi) Bosch from which it differs by shorter leaves, 1–4 sori per pinna, and campanulate immersed involucres with wide-flaring apex. This species is always epiphytic while *T. rupestre* is epipetric or terrestrial, but not epiphytic. This species is dedicated to Ramón Riba y Nava Esparza.

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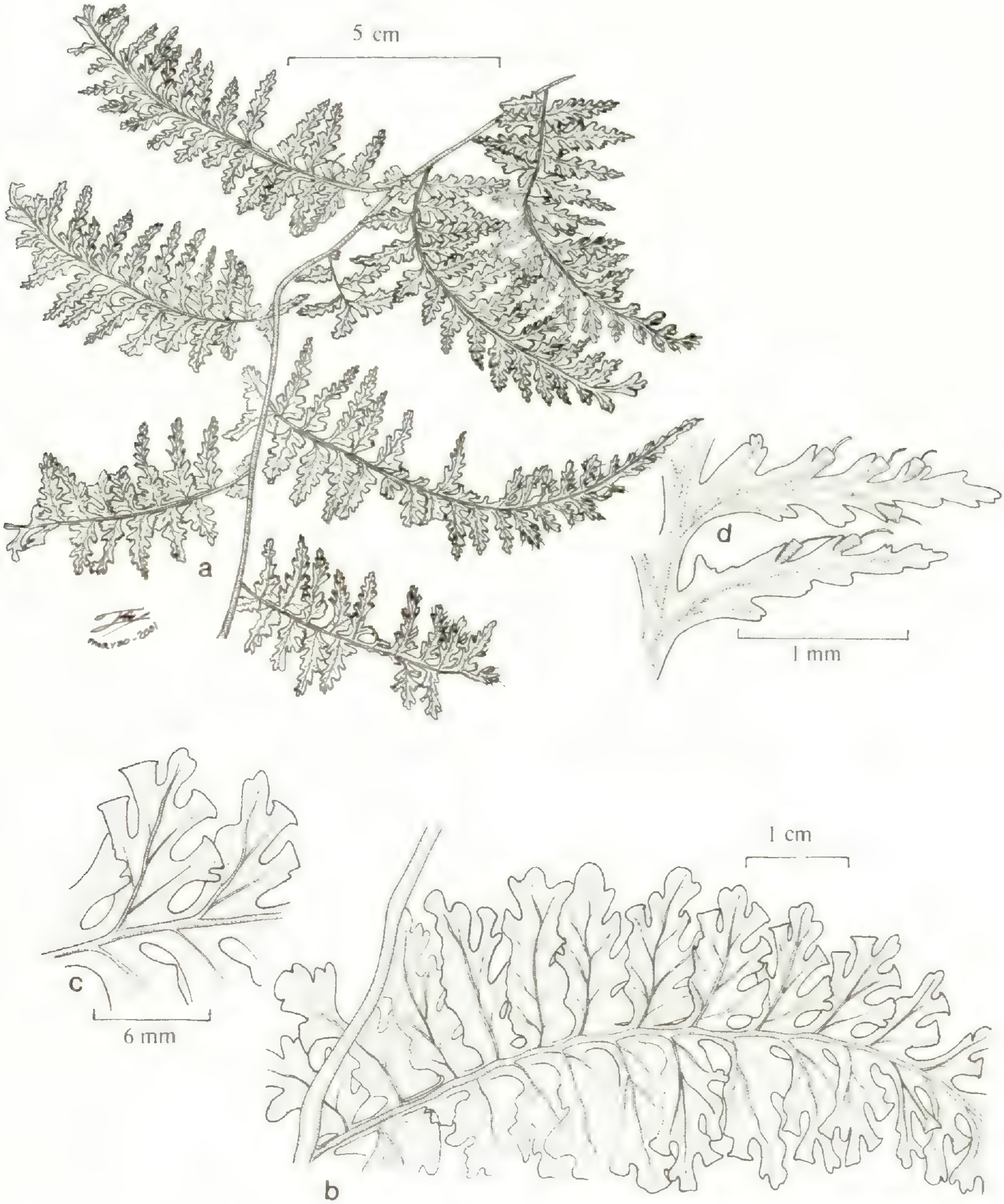

FIG. 1. *Trichomanes ribae.* a) habit; b) general view of leaf; c) pinna with campanulate immersed involucres with wide-flaring apices. *Trichomanes rupestre.* d) pinna with involucres.

Jiménez for the drawings that illustrate this new species. I am also grateful to Fernando Chiang for the Latin translation of the species diagnosis.—LETICIA PACHECO. Departamento de Biología-Botánica Estructural y Sistemática Vegetal, Universidad Autónoma Metropolitana-Iztapalapa, Apdo. Postal 55-535, 09340 México D. F., México.

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Referees for 2002

All papers submitted to the journal are peer reviewed. Members of the editorial board and the Society, as well as additional scientists in cognate areas, do these reviews on a voluntary basis. It is their work that contributes to the high quality of articles in the American Fern Journal and to its continued success. The American Fern Society and I extend our thanks to the following reviewers for their assistance, diligence, and patience in the year 2002. Special thanks are given to Dr. David B. Lellinger for assuming the task of editing the Wagner Memorial Issue—R. James Hickey.

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